Mechanisms underlying the role of sex hormones in the pathogenesis of rheumatoid arthritis

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MECHANISMS UNDERLYING THE ROLE OF SEX HORMONES IN THE PATHOGENESIS OF RHEUMATOID ARTHRITIS

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ABBREVIATIONS

APAAP	Alkaline phosphatase-antialkaline phosphatase
APC	Antigen presenting cell
AR	Androgen receptor
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CBG	Corticosteroid binding globulin
CFU-GM	Colony-forming unit, granulocyte-macrophage
CIA	Collagen-induced arthritis
ConA	Concanavalin A
CPJ	Cartilage/pannus junction
CS-FCS	Charcoal-stripped foetal calf serum,
CTP	Cytosine triphosphate
DEPC	Diethylpyrocarbonate
DES	Diethylstilbestrol
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
DHT	Dihydrotestosterone
DMSO	Dimethylsulphoxide
(c)DNA	(complementary) Deoxyribonucleic acid
DTH	Delayed-type hypersensitivity
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ER	Oestrogen receptor
ERE	Oestrogen responsive element
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
FSH	Follicle-stimulating hormone
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GH	Growth hormone
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GnRH	Gonadotropin-releasing hormone
GR	Glucocorticoid receptor
GTP	Guanosine triphosphate

HCG	Human chorionic gonadotropin
HRE	Hormone responsive element
Hsp	Heat shock protein
IFN	Interferon
IL-1 (-2,-3,-4,-6,-8)	Interleukin-1 (-2,-3,-4,-6,-8)
IRAP	Interleukin-1 receptor antagonist protein
LH	Luteinising hormone
LHRH	Luteinising hormone-releasing hormone
LPS	Lipopolysaccharide
LT	Lymphotoxin
MAb	Monoclonal antibody
MCM	Monocyte conditioned medium
βME	β-Mercaptoethanol
MEM	Minimal Essential medium
MFI	Mean fluorescence intensity
MLR	Mixed lymphocyte reaction
MNC	Mononuclear cell
MOPS	3-[N-Morpholino]propanesulfonic acid
MTT	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide
NK cell	Natural killer cell
NRS	Normal rabbit serum
NSAID	Non-steroidal anti-inflammatory drug
OA	Osteoarthritis
ODC	Ornithine decarboxylase
OP	Osteoporosis
PAO	Polyamine oxidase
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGE_2	Prostaglandin E_2
PHA	Phytohaemagglutinin
PKC	Protein kinase C
PMNL	Polymorphonuclear leukocyte
PR	Progesterone receptor
PWM	Pokeweed mitogen
RA	Rheumatoid arthritis
(m)RNA	(messenger) Ribonucleic acid

R-PE	R-Phycoerythrin
RT	Reverse transcriptase
20αSDH	20α hydroxysteroid dehydrogenase
SDS	Sodium dodecyl sulphate
SHBG	Sex hormone binding globulin
SLE	Systemic lupus erythematosus
SSC	Side scatter
TAE	TRIS-acetate-EDTA
TBE	TRIS-borate-EDTA
TBS	TRIS buffered saline
TEMED	N,N,N',N',-Tetramethylethylenediamine
TGF	Transforming growth factor
TNF	Tumour necrosis factor
TTP	Thymidine triphosphate

SUMMARY

Rheumatoid arthritis (RA) is an inflammatory, autoimmune condition, the pathogenesis of which is unclear. The disease is characterised by an infiltration of inflammatory cells into the synovium and subsequent release of factors such as cytokines, which have the capacity to both trigger events leading to destruction of the joint architecture, and also mediate the perpetuation of the inflammatory process, for example by modulating the expression of adhesion molecules, thus suggesting a crucial role for the immune system. Females are known to have a heightened immune response compared to males, and also an increased susceptibility to autoimmune disorders, including RA, the onset and severity of which can be modulated by periods of endogenous hormonal changes, indicating a possible association with the sex hormones.

The aim of this Ph.D. was to investigate the effect of sex hormones on the immune system *in vitro*, using peripheral blood mononuclear cells (PBMC) from RA patients and from normal, healthy controls. Various parameters were assessed, including cytokine production and integrin expression, with particular reference to changes in immune function thought to occur in RA.

Oestrogen and testosterone had no effect on the release of interleukin-6 (IL-6) or tumour necrosis factor (TNF) from control PBMC. In addition, oestrogen had no effect on interleukin-1β (IL-1β), IL-6, TNF-α, transforming growth factor-β (TGF-β) or prolactin mRNA expression, using either control or rheumatoid cells. PBMC proliferation was also unaffected by oestrogen and testosterone, whereas the same concentration range of oestrogen induced significant proliferation of an oestrogen-dependent cell line, ZR-75. The morphology of ZR-75 cells was altered by culturing in the absence of oestrogen, which correlated with a downregulation of the integrin subunit, α6. However, incubation of control or rheumatoid PBMC with and without oestrogen did not alter their pattern of integrin subunit expression.

Oestrogen receptor (ER) protein and mRNA were readily detectable in the ZR-75 cell line, whereas the receptor protein was undetectable in all other cell types, including PBMC, and the mRNA was present in only very low amounts. The expression of p29 antigen, an ER related protein, was demonstrated in all cell types studied, including PBMC, synovial fibroblasts and rheumatoid synovial tissue. This data suggests that the sex hormones, oestrogen and testosterone are ineffective in *in vitro* assays of immune function, when studying control or rheumatoid PBMC, which may be a result of constitutively low receptor levels.

CHAPTER ONE

INTRODUCTION

1.1 AN OVERVIEW OF RHEUMATOID ARTHRITIS, THE IMMUNE SYSTEM AND SEX HORMONES

Rheumatoid arthritis (RA) is an autoimmune inflammatory disease which is associated with destruction of the articular cartilage, in conjunction with bone and soft tissue. In RA, the synovial membrane becomes hypertrophied as a result of the infiltration of cells, mainly lymphocytes and monocytes, and the local proliferation of synovial fibroblasts. Neovascularisation also occurs, leading to the formation of a pannus which grows over and erodes the surface of the cartilage and subchondral bone. Susceptibility to the disease appears to be linked to the class II MHC alleles HLA-DR4 and -DR1. However, the actual trigger for disease onset remains a hypothetical issue and other factors are likely to modulate the severity.

The infiltration of the synovial tissue with cells which have the capacity to elicit an inflammatory response indicates that this process is central to the pathogenesis of RA. In the normal situation, inflammatory reactions are controlled, with factors released concomitantly to both upregulate and downregulate cell responses. The release of interleukin-1 (IL-1) from accessory cells activates T cells which release cytokines such as interleukin-2 (IL-2) and interferon-γ (IFN-γ). IFN-γ has the capacity to activate monocytes and macrophages to release further IL-1 and other monokines such as tumour necrosis factor-α (TNF-α). Hence, a cascade of inflammatory mediators is generated. However, in a disease such as RA, there is a failure to contain the inflammatory response and a chronic, destructive scenario arises. Within the rheumatoid joint, T cells appear in a pseudo-activated state, displaying an upregulation of certain activation markers on their cell surface, whilst the production of IL-2 and IFN-γ and the expression of IL-2 receptors are downregulated. It is thought that macrophages, synoviocytes and chondrocytes are the main effector cells, with the capacity to produce an array of cytokines, growth factors and degradative enzymes, all of which are detectable in significant amounts within the RA synovial membrane and fluid. The factors released are potentially responsible for both the destruction seen within the joint and also reparative processes which occur in parallel.

The mechanism of cellular infiltration into the RA synovial membrane is thought to be via adhesion molecules, including the integrin family, the expression of which is modulated by the inflammatory process. Systemic and local release of cytokines following the initiation of an inflammatory response is likely to upregulate ligands, such as ICAM-1, ELAM-1 and VCAM-1 on endothelial cells, and their respective receptors on the surface of circulating cells, thus leading to an increase in cellular

adhesiveness. Trans-endothelial migration and the perpetuation of cells within the joint is also thought to be mediated by integrins, with the latter occurring as a result of binding to connective tissue components and cell-cell interactions.

The presence of cells such as T cells and monocytes within the synovial tissue and the detection of mediators released by these cells which have the potential to both trigger the destructive processes occurring in RA and participate in the upregulation and perpetuation of the inflammatory response, for example by increasing expression of adhesion molecules, indicates that the immune system is crucial to the development and progression of RA. Research into causes of, and potential therapy for, the disease have therefore centred around immune function. A relationship between gender, sex hormones and the immune system has been recognised for many years. Females have a heightened humoral and cell-mediated immune response compared to males, a greater resistance to infections, increased tumour rejection capacity and a relative resistance to tolerance. In addition, women are more likely to develop autoimmune diseases than are men. In RA, the female:male ratio is approximately 3.5:1 with disease onset occurring predominantly at the time of the menopause. Pregnancy often results in a clinical remission although exacerbation is seen in as many cases postpartum. Thus, *prima facie*, there would seem to be an association between the immune system, autoimmunity and sex hormones.

In the case of systemic lupus erythematosus (SLE), an autoimmune condition characterised by anti-dsDNA and other antibody formation, inflammation and damage to the kidneys and central nervous system, women, especially those of child-bearing age, are approximately ten times more likely to develop the disease. In addition, other autoimmune conditions including scleroderma, autoimmune diabetes mellitus and Sjogren's syndrome also affect predominantly the female sex. Other arthritides, including osteoarthritis (OA), and bone disorders, such as osteoporosis (OP), also seem to be hormonally linked. OA occurs predominantly in females around the time of the menopause and has been related to a period of hormonal imbalance, rather than being due to simple 'wear and tear' as originally thought (reviewed by Spector & Campion, 1989). Osteoporotic fractures are seen in 1:2 females but in only 1:40 men, with an increase in the incidence from the seventh decade onwards. OP has been linked to the loss of ovarian function and its onset is effectively delayed by the administration of female sex hormones (reviewed by Hillard & Stevenson, 1991; Lindsay, 1991).

The role of the immune system in RA and the potential association with sex hormones has resulted in an increasing interest in the role of these hormones in immune function.

Many of the initial investigations were centred around the effect of natural fluctuations in endogenous hormone levels, and the effect of altering these levels, such as during pregnancy, at the time of the menopause and with the use of oral contraceptives, in relation to disease onset and activity. With the explosion of knowledge into the many aspects of immunity in recent years, studies have become geared to a role for sex hormones in specific aspects of the immune system which may in turn explain their role in RA. However, pharmacological doses of the hormones are often employed in studies of cell function *in vitro*, and one may question the relevance of this to the *in vivo* situation.

The following review of the literature will begin by discussing the clinical and epidemiological studies of sex hormones and the part they play in the pathogenesis of RA, after a brief introduction into the production and metabolism of the sex hormones and their receptors. Secondly, functional studies of sex hormones and the immune system will be addressed, including the role of specific immune cell types, cytokines released and adhesion molecules, in relation to the pathogenesis of RA as described above. Comparisons are made between RA and SLE, as the hormones often appear to have differing effects in these autoimmune diseases, which may be useful when trying to unravel the complexities of sex hormones and the immune system.

1.2 SEX HORMONES AND THEIR RECEPTORS

1.2.1 Production and Metabolism

Sex hormones are derived from cholesterol, with testosterone and oestradiol being the major biologically active species (reviewed by Siiteri, 1979; Spector, 1989). The various routes of hormone production and metabolism are summarised in Figure 1.1. In men, testosterone is mainly derived from the testes (approximately 80%), with the remainder originating from the adrenal glands. Testosterone is metabolised to dihydrotestosterone (DHT) and to oestradiol, with a combination of all three hormones being responsible for its biological effects. Oestradiol is formed from testosterone, as described above, and also from the conversion of androstendione to oestrone. In females, 30-40% of the total testosterone produced is from the peripheral conversion of serum androstenedione, approximately 25% is from the ovaries, with the remainder originating from the adrenals.

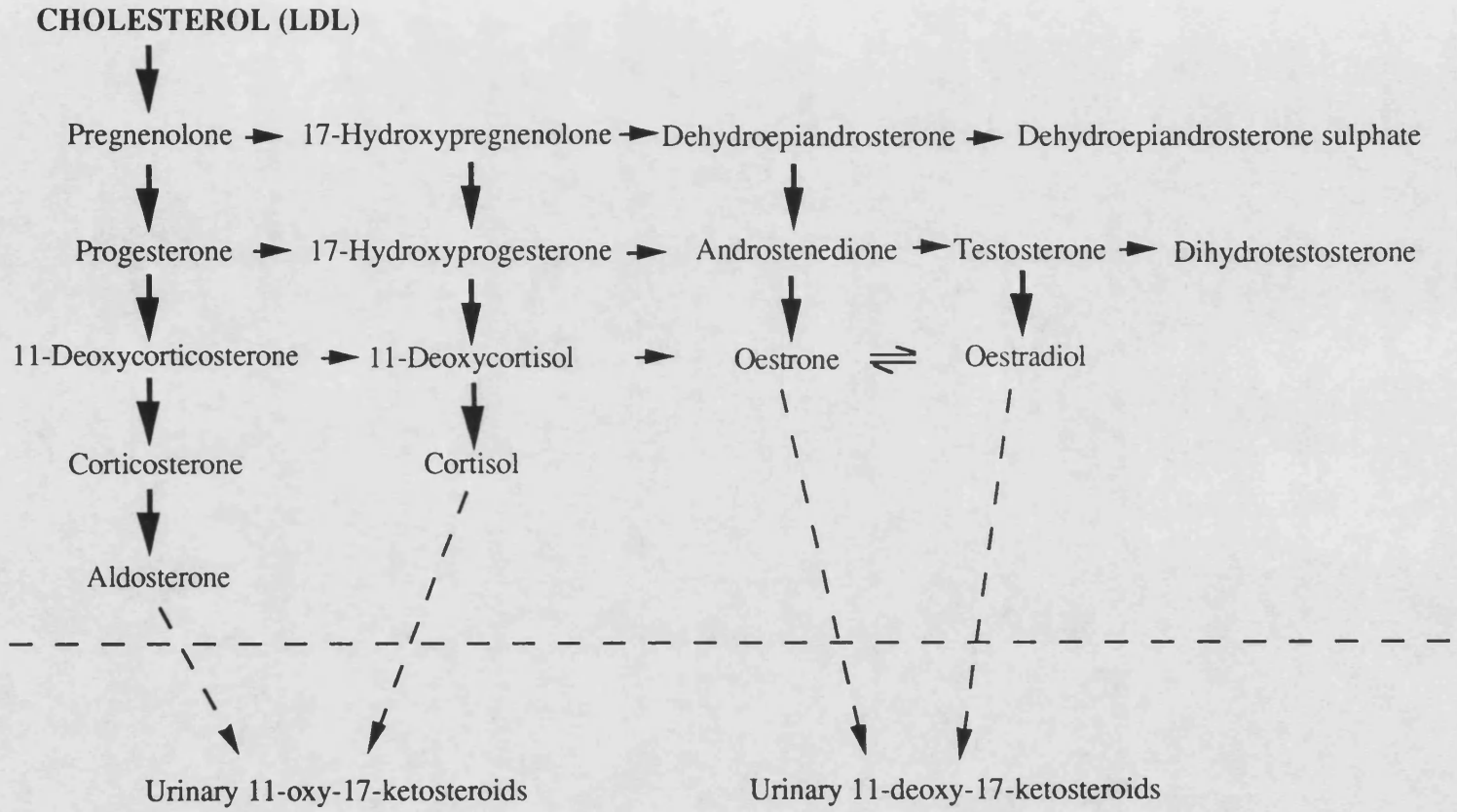


Figure 1.1 Primary routes of biosynthesis and metabolism of the major sex steroids. (Spector, 1989).

The majority of circulating oestradiol in females derives from the follicle destined to ovulate. Extra-glandular formation of oestrogen from circulating androgens is a principal source of oestrogen, not only in males, but also in postmenopausal women. The amount formed depends on the precursor availability and metabolic factors such as obesity and hepatic disease. During pregnancy up to 50% of dehydroepiandrosterone sulphate (DHEAS) is converted to oestradiol in the placenta which helps to maintain high levels of this hormone.

The secretion of testosterone is controlled by luteinising hormone (LH) produced by the pituitary, which in turn is under the control of luteinising hormone-releasing hormone (LHRH) and factors such as circulating testosterone levels. Oestrogen secretion is controlled by follicle-stimulating hormone (FSH) and also by positive and negative feedback mechanisms relating to plasma levels. As LHRH can stimulate the release of FSH as well as LH, and a separate follicle-stimulating hormone releasing-hormone has yet to be defined, LHRH is sometimes referred to as gonadotropin-releasing hormone (GnRH).

Steroid hormones in the blood are reversibly bound to albumin and other proteins, including corticosteroid binding globulin (CBG) and sex hormone binding globulin (SHBG), a glycoprotein of molecular weight 94 kD. The binding to albumin is weak and non-specific, whereas binding to CBG and SHBG is of much higher affinity and is specific. CBG binds cortisol and progesterone and SHBG binds androgens and oestrogens with affinity: DHT > testosterone > oestradiol. The binding capacity of SHBG is twice as great in females and oestrogens are able to stimulate, and androgens suppress, SHBG synthesis. The result of this is that only approximately 2-3% of the total steroid in serum occurs in a free form, available for target cell receptors. However, the relevance of SHBG in sex hormone physiology is still unclear.

1.2.2 Receptors

To assess the sensitivity of a given cell type or tissue to the effects of sex hormones it is essential to first ascertain whether or not specific receptors are present. The development of radioactively labelled ligands enabled specific binding sites to be detected. Subsequent to this, isolation of cDNA for the receptors has identified a family of related genes which bind ligands of great diversity, including the adrenal steroids, sex steroids and vitamin D_3 (reviewed by Green & Chambon, 1986; Evans, 1988). The initial cloning of the human glucocorticoid receptor (GR) demonstrated an incredible

similarity to the viral oncogene *erb*A (Hollenberg *et al.*, 1985). This relationship was confirmed for other receptors including that for oestrogen (ER) (Green *et al.*, 1986). In addition, the *erb*A proto-oncogene product was identified as the thyroid hormone receptor (Weinberger *et al.*, 1986). Thus, the steroid and thyroid hormone receptor superfamily was defined, and is continuing to expand as more gene products are identified. Two such products are ER-related genes 1 and 2 (ERR1 and ERR2), the ligands for which are as yet unknown, hence the term 'orphan receptors' is used (Giguere *et al.*, 1988).

ER was identified within rat uterus, associated with the nuclear and cytosolic components (Toft & Gorski, 1966). This led to the design of a two-step model for oestrogen action within the cell, whereby cytoplasmic ER, upon binding oestrogen, could translocate to the nucleus and activate specific genes (Jensen *et al.*, 1968). However, the subsequent development of immunocytochemical techniques allowed receptors to be studied *in situ*, which demonstrated that the unoccupied ER resided in the nucleus, and that the previously shown cytosolic form was in fact due to disruption techniques employed during experimental procedures (King & Greene, 1984). Thus, a new model was formulated in which the unoccupied form of the ER resided in the nucleus, loosely associated with certain genomic elements, and subsequent hormone binding resulted in a stronger association with these elements and hence the initiation of transcription (Greene & Press, 1986). This model was also confirmed for other members of the steroid and thyroid hormone receptor family. However, some receptors, such as that for glucocorticoid, are cytoplasmic in nature. This is thought to be due the presence of nuclear localisation domains within the GR which are inactive in the absence of ligand. In the case of ER the nuclear localisation signal is constitutively active (Picard *et al.*, 1990a).

Functional domains

The comparison of sequences for the sex steroid receptors along with mutation experiments have revealed that functional domains exist, with each domain being responsible for a specific step in receptor activation. Figure 1.2 demonstrates the functional domains of the ER. Kumar *et al.* (1986; 1987) constructed a series of human ER deletion mutants using site-directed mutagenesis, and transfected these into HeLa cells, which do not express ER constitutively. They confirmed that the highly conserved regions C and E were responsible for DNA- and ligand-binding, respectively. In addition, they proposed that region D, whose length and composition

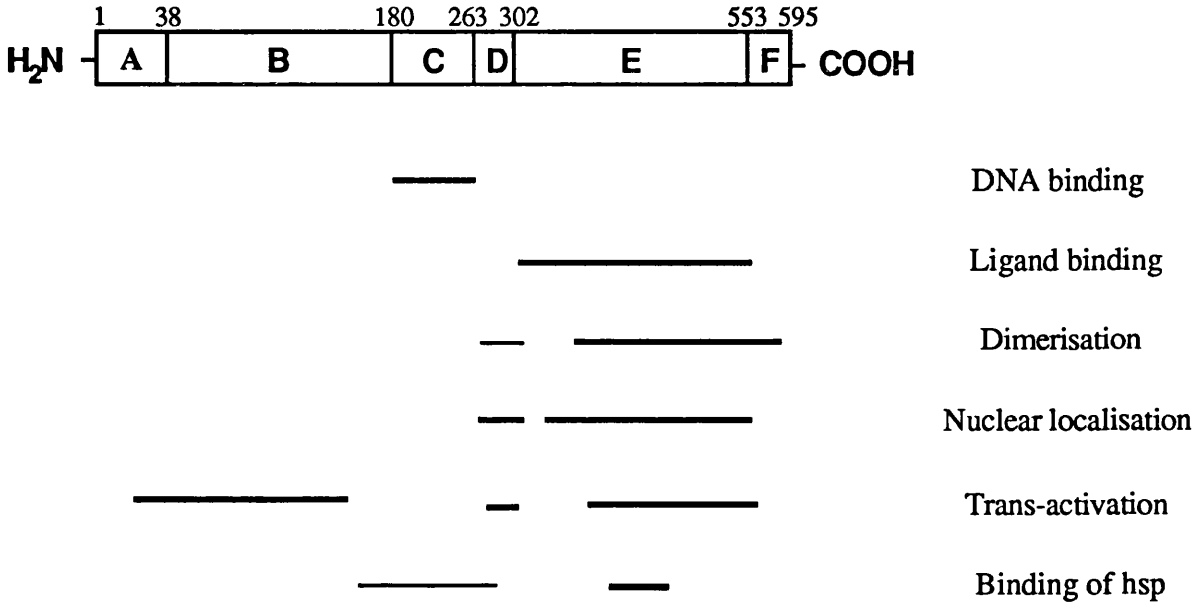


Figure 1.2 Functional domains of the oestrogen receptor.

did not affect receptor functioning, could act as a hinge between regions C and E, and region A/B may be necessary for effective transcription in some oestrogen-responsive genes. The mechanism of hormone-regulated gene expression has been widely reviewed (Beato, 1989; Rories & Spelsberg, 1989; Gibson *et al.*, 1990; Parker, 1990; Wahli & Martinez, 1991; King, 1992).

The DNA-binding domain was found to be cysteine-rich and thus able to form two 'fingered' structures, CI and CII, coordinated by a zinc ion. These zinc fingers thus had the potential to interact with the DNA helix of a specific region of the genome, known as a hormone responsive element (HRE). Receptor species are thought to be the same in different tissues, therefore it is the difference between these nuclear binding sites which is likely to determine a tissue-specific response of a given hormone. The first, N-terminal, finger was shown to be responsible for specific DNA sequence recognition, with the second likely to be involved in non-specific DNA binding and hence stabilisation of the complex (Green *et al.*, 1988). In particular, it is the three amino acids on the C-terminal side of CI which confer sequence specificity (Mader *et al.*, 1989). Thus, binding of hormone-receptor complexes, the *trans*-acting proteins, to *cis*-acting HRE sites on the DNA, via region C, culminates in transcriptional regulation of hormone-responsive genes.

The sequences of specific HREs have now been defined. Klein-Hitpass *et al.* (1988) found that the 13 bp oestrogen responsive element (ERE) of the *Xenopus* vitellogenin A2 gene differed from that of the A1 and the chicken vitellogenin II genes by only three central positions, and hence they derived the consensus ERE: GGTCANNNTGACC. An ERE has since been demonstrated in human genomic DNA (Inoue *et al.*, 1991) and EREs have also been reported in the 5' flanking region of both the human GnRH gene (Radovick *et al.*, 1991) and the rat prolactin gene (Maurer & Notides, 1987), and within the rabbit progesterone receptor (PR) gene (Savouret *et al.*, 1991). The similarity between the sequences of the ERE and responsive elements for other hormones, such as glucocorticoid and progesterone, means that substitution of just one or two base pairs can be sufficient to convert one response element to another (Klock *et al.*, 1987). Interestingly, a protein capable of binding region II of the mouse MHC class I regulatory element (H-2RIIBP) showed homology with the DNA-binding domains of hormone receptors and was found to bind to an ERE (Hamada *et al.*, 1989). There is also the potential for 'cross-talk' whereby heterodimers are formed between two subunits not normally associated, leading to an inhibition of a response through a specific enhancer sequence (reviewed by King, 1992). A complex network

of interactions arises due to the conservation of both the structure of DNA-binding domains and also homology of responsive elements (reviewed by Beato *et al.*, 1989).

Sequencing of the HREs of the steroid hormone receptors revealed them to be palindromic elements containing 5-6 bp in each half of the palindrome, separated by 3 bp (see Klock *et al.*, 1987). It was speculated therefore, that the hormone-receptor complex would bind as a dimer. Indeed, it was found that the activated receptor, with bound ligand, formed a homodimer via noncovalent interactions between the two C and the two E regions, with the two C regions held together such that they could recognise and bind the HRE simultaneously (Kumar & Chambon, 1988). The necessity of a ligand-binding domain for receptor dimerisation and DNA-interaction is somewhat controversial, with some reports stating that hormone-binding is essential for the formation of stable dimers (Kumar & Chambon, 1988) and effective gene transcription (Kumar *et al.*, 1987; Webster *et al.*, 1988), whereas others have reported that ER was able to bind an ERE in the presence or absence of oestrogen, dependent on Mg^{2+} concentrations and temperature (Brown & Sharp, 1990), as a monomer or a dimer (Medici *et al.*, 1991).

One possible explanation for the contradictory literature is the association of the receptor with a heat shock protein of 90 kD (hsp90). Hsp90 is a non-DNA, non-steroid binding phosphoprotein which has been identified as a component of non-transformed 8S receptors of steroid hormones including progesterone, oestrogen, androgen and glucocorticoid receptors (Catelli *et al.*, 1985). In addition, the production of certain heat shock proteins, such as the 90 and 108 kD types, is dependent on sex hormones, and their production is increased following acute or chronic stimulation with oestrogen and/or progesterone (Ramachandran *et al.*, 1988). Following studies with the GR, it was hypothesised that the free hormone-binding domain complexes with hsp90, thus preventing DNA-binding, with the binding of ligand subsequently relieving this inhibition (Denis *et al.*, 1988; Picard *et al.*, 1988). This was further supported by the observation that deletion of the hormone-binding domain of ER inhibited interaction with hsp90 (Green, 1990). Picard *et al.* (1990b) demonstrated that reduced levels of hsp90 inhibited GR transcriptional activity. However, Kumar *et al.* (1987) found that by removing the hormone-binding domain of the ER, thus inhibiting association with both ligand and hsp90, weak binding to the ERE could still occur. The interaction between the ER and hsp90 was shown to require sequences N-terminal to the ligand-binding domain, as well as region E itself (see figure 1.2) (Schlatter *et al.*, 1992). Thus, in studies utilising a receptor which is already dissociated from hsp90, it may be expected that DNA-binding would occur in the absence of hormone.

In general, it would seem that the presence of hormone is essential for gene regulation. It has been suggested that DNA and oestrogen may act together to transform the ER into the correct conformation for transcriptional activation, as DNA-binding altered the dissociation of oestrogen from the receptor complex (Fritsch *et al.*, 1992). A similar theory of conformational change, this time by folding of the ERE into a structure which could be bound more tightly, was proposed by Lannigan & Notides (1989). Synergism between two ERE with individually low enhancer activities, resulting in a complex with high oestrogen inducibility has also been reported (Klein-Hitpass *et al.*, 1988). The synergism was most efficient with ERE in close proximity, and was hypothesised to occur as a result of short range DNA conformational changes. Thus, it may not be the sequence of the HRE alone which confers specificity, but also the three dimensional configuration of the DNA. The ER also requires phosphorylation for effective activation, and conversely, dephosphorylation for inactivation. This was initially thought to occur on tyrosine residues, but more recently it is the serine residues which have been shown to be the primary target (Denton *et al.*, 1992), suggesting a role for protein kinase C (PKC) which is a serine-threonine kinase. It was proposed that phosphorylation would increase the affinity of the receptor for DNA sequences and therefore prolong the life of the ER/ERE complex.

Oestrogen is known to activate transcription by means of two intra-receptor sequences, TAF-1, in the A/B region, and TAF-2, in the E/F region, as well as via the standard transcription-activation function of the DNA-binding domain (Tora *et al.*, 1989). TAF-1 is constitutively active whereas TAF-2 requires oestrogen-binding. It is thought that the anti-oestrogen, tamoxifen, acts as a partial agonist by the promotion of the DNA-binding of an ER in which TAF-1 is active and TAF-2 is not (reviewed by Green, 1990). In contrast, the full antagonist, ICI 164,384, binds the receptor and induces a conformational change such that the complex can bind to the ERE, but fails to promote subsequent transcriptional events (Sabbah *et al.*, 1991).

Steroids can regulate gene expression at a transcriptional level or post-transcriptionally, by affecting events such as mRNA processing and stabilisation, protein synthesis, processing and secretion. As well as activating genes by binding to enhancer sequences on the DNA, hormone receptors can mediate repression by binding silencers. This may occur by the attachment to a different HRE, or in a competitive nature by binding to an overlapping DNA sequence. Alternatively, a region other than the DNA-binding site may be involved (reviewed by Beato, 1989). Steroid induction of transcription requires numerous protein factors in addition to the primary requirement for an interaction of the active receptor complex with its HRE.

Figure 1.3 represents a possible mechanism for gene transcription (reviewed by Rories & Spelsberg, 1989). Initially, the activated steroid receptor complex (R) binds to an acceptor site (A), which then becomes associated with the nuclear matrix (NM), or binds an acceptor site which is already linked to the nuclear matrix. This then enables the DNA-binding domain of the receptor to interact with an HRE which may occur at some distance from the acceptor site, and other transcription factors, denoted TF1 and TF2, are then able to bind, with DNA folding occurring to bring the enhancer and promoter sites into closer proximity. Finally, the protein-DNA complex thus formed is recognised by RNA polymerase II, additional transcription factors bind and gene transcription ensues. The complex remains in contact with the nuclear matrix as this contains the hormone responsive genes.

1.3 THE INFLUENCE OF SEX HORMONES IN RHEUMATOID ARTHRITIS

1.3.1 Abnormalities in Sex Hormone Metabolism

Various clinical and epidemiological studies have been carried out in search of a link between aberrant sex hormone metabolism or natural hormonal fluctuations and autoimmunity. The results of studies of hormone levels in female patients have been variable. Reductions in oestrone, DHEAS and testosterone levels have been recorded in postmenopausal women, possibly as a result of corticosteroid therapy (Sambrook *et al.*, 1988), whereas a further study reported increases in testosterone, androstenedione and DHEAS postmenopausally (Cutolo *et al.*, 1986). An increase in cortisol levels in both pre- and postmenopausal patients has also been observed, possibly as a result of the stress and pain incurred (Spector *et al.*, 1987). Thus, there is a great deal of conflicting evidence for hormonal abnormalities in female RA sufferers.

The consistent finding however, is of low testosterone levels in male RA patients. Studies which formed this conclusion measured either serum total (ie. bound and unbound) testosterone (Cutolo *et al.*, 1988), or both total and free (ie. unbound) levels (Gordon *et al.*, 1988; Spector *et al.*, 1988; Swinden *et al.*, 1990). Free, unbound testosterone is believed to be the principal active androgen and is therefore a more reliable indicator of androgen status than total serum testosterone alone. Gordon *et al.* (1988) measured testosterone levels before, during and after rheumatoid flares in ten male patients and concluded that this peak in disease activity was associated with prolonged suppression of testicular function. The reductions in testosterone reported do

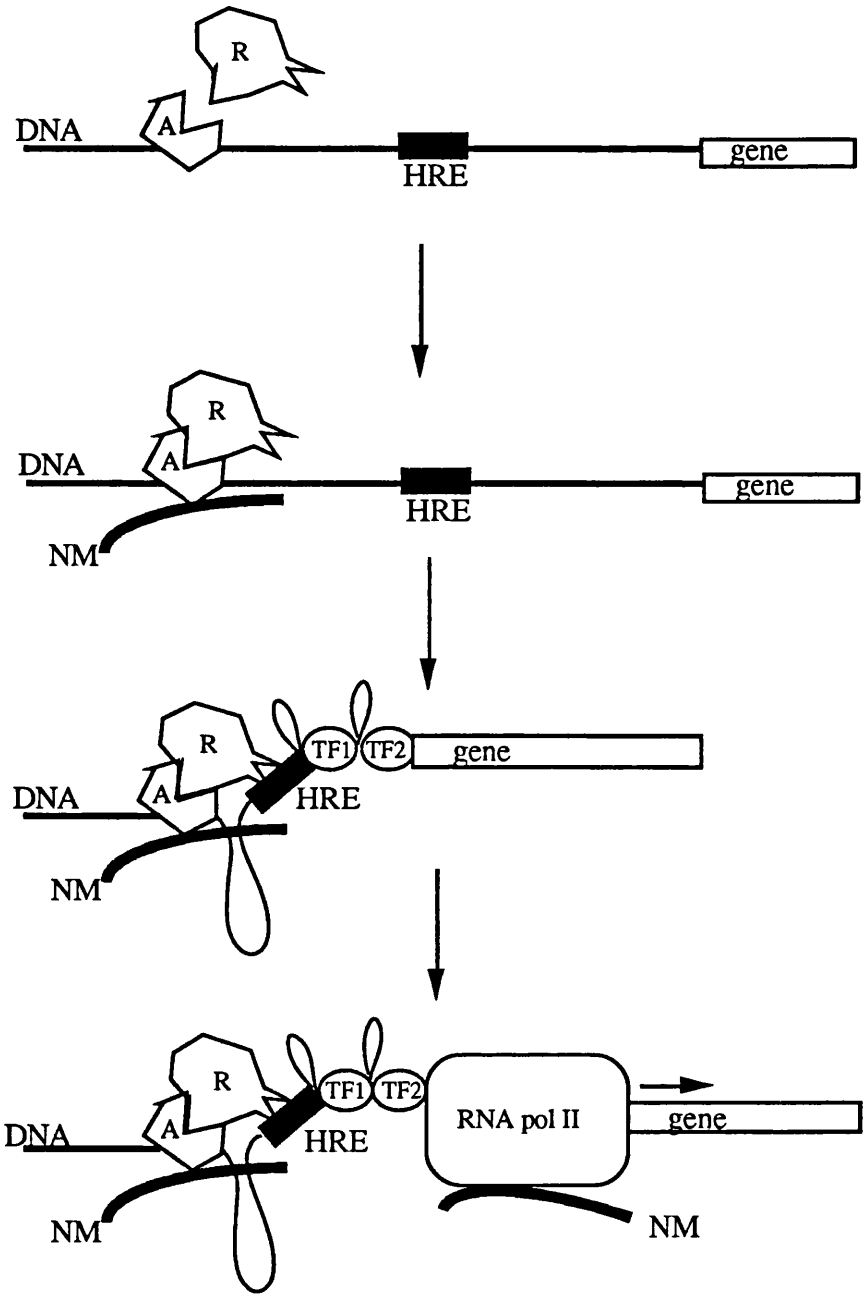


Figure 1.3 A model of steroid receptor action on gene transcription (Rories & Spelsberg, 1989). See text for details of abbreviations.

not appear to be due to abnormalities in the hypothalamic-pituitary axis in that responses to LH and FSH are as expected (Cutolo *et al.*, 1988; Gordon *et al.*, 1988), except for one study, which found no increase in LH in response to the lowered testosterone (Spector *et al.*, 1988). However, the latter occurred in both the RA group and the OA controls, and is therefore more likely to be a result of chronic disease *per se*. A lowered response to human chorionic gonadotropin (HCG), which stimulates testicular testosterone production, was noted in one investigation, perhaps indicating a reduced testicular steroid biosynthesis pool or chronically low gonadotropin stimulation (Cutolo *et al.*, 1988).

Evidence suggests that there may also be a link between the HLA system, sex hormones and RA (reviewed by Spector, 1989; Cutolo & Accardo, 1991). An association between RA and the class II antigen DR4 is widely reported (Panayi *et al.*, 1978). Studies have shown a link between testosterone levels and the HLA-B locus (Gerencer *et al.*, 1982), and the enzyme 21-hydroxylase, responsible for synthesis of both cortisol and sex steroids, has been mapped to within the class III region of chromosome 6, close to the B locus (New, 1985). It has been speculated that gene(s) within the HLA region could therefore either influence androgen metabolism directly, for example via 21-hydroxylase, or perhaps class I and II molecules, important in antigen presentation to T cells, interact with specific androgen receptors on specialised antigen presenting cells (APC) (reviewed by Cutolo & Accardo, 1991). In the mouse, several loci in the MHC control both the immune system and various aspects of sex differentiation, including blood testosterone and testosterone binding globulin levels and sensitivity to testosterone (Ivanyi *et al.*, 1972). Thus, in genetically susceptible individuals, there may by abnormal hormone metabolism which leads to, or exacerbates, RA. However, it may be the case that alterations in hormonal levels are a consequence of the disease and/or treatment rather than being the predisposing factor.

If the oestrogen/androgen story were to be taken literally then it could be concluded that women are protected by endogenous hormones up to the onset of the menopause when onset of RA is most likely to occur. In males there is an inverse relationship due to declining androgen levels with age. Any event which prematurely upsets the balance of hormones is thus likely to lead to onset of disease in susceptible individuals. However, as will be seen in the proceeding sections, events which cause considerable change to the body's natural hormonal pattern, such as pregnancy and the ingestion of oral contraceptives, do not have such clear-cut effects, and the endocrine-immune association seems to be far more complicated than anticipated (reviewed by Spector, 1989; DaSilva & Hall, 1992).

1.3.2 The Menstrual Cycle and the Menopause

A recent report has shown that the average female develops her first symptoms of RA at the time of her menopause (McHugh, 1990). Indeed, the female:male ratio in RA reaches a peak at the age of 40-44 years, then declines to unity after the sixth decade of life (Goemaere *et al.*, 1990). Although the effects of dramatic hormonal fluctuations at the time of the menopause on RA appear to be very clear cut, relatively moderate cyclical hormonal changes, such as those which occur during the menstrual cycle, seem to have less obvious effects. There have been reports of differences in disease activity at various stages of the cycle (Latman, 1983; Rudge *et al.*, 1983; Grinlinton, 1988). Reductions in severity were recorded during the postovulatory phase, when plasma concentrations of progesterone and oestrogen are high (Latman, 1983), and increases in clinical measurements of RA disease activity were detected in the late-luteal phase compared to the mid-follicular phase, following these hormonal surges (Grinlinton, 1988). However, others have failed to demonstrate any effect of the menstrual cycle on RA (Goldstein *et al.*, 1987).

1.3.3 Oral Contraceptive Use

Data regarding the use of oral contraceptives and RA needs careful analysis due to the influence of a variety of parameters. First, studies are either of the cohort type, whereby individuals without RA are followed and examined for the later development of the disease, or are case-control studies in which RA patients are compared to controls without RA for past and present use of oral contraceptives. Secondly, the choice of controls for case-control studies is an important consideration, as is the date of the study, whether it be of the cohort or case-control type, its location and the collection of data following the survey. Attempts have been made to clarify the potential confounding issues (Esdaile, 1989).

In two cohort studies, one from the UK and one from the USA, no protection from oral contraceptive use was seen (Hannaford *et al.*, 1990; Hernandez-Avila *et al.*, 1990). The former group summarised the data from a large study originating from the Royal College of General Practitioners which began in 1968. The data available in 1978 suggested a halving of the risk for RA in current users (Royal College of General Practitioners, 1978), but by 1987 the benefit seemed to have disappeared. It was suggested that a reduction in the incidence of RA amongst former and never users between 1968 and 1987, with no such trend seen with current users of oral

contraceptives, meant that the ratio between current and never users had approached unity. Another possible explanation is that the change from high oestrogen to low oestrogen oral contraceptives resulted in the loss of a protective effect.

Other studies however, have reported a protective effect of oral contraceptive use to date in RA (Vandenbrouke *et al.*, 1982; Vandenbrouke *et al.*, 1986; Hazes *et al.*, 1989a; Koepsell *et al.*, 1989; Hazes *et al.*, 1990a; Hazes *et al.*, 1990b; Spector *et al.*, 1990). This includes protection with current but not past use (Koepsell *et al.*, 1989), a reduction in RA but only with oral contraceptive use before the age of 35 (Spector *et al.*, 1990) or greater protection in females with a family history of RA and those aged 31-40 years at the onset of the disease (Hazes *et al.*, 1990b). Alternatively, there may simply be a protective effect reported, independent of age or whether use is past or present. To overcome the problem of adequate control selection, Hazes *et al.* (1989b; 1990a) used female siblings, either sisters also suffering from RA in multicase families, or unaffected sisters from sporadic case families. These results showed a protective effect which was much stronger in the multicase than the sporadic case families. In a recent review it was concluded that oral contraceptives may mitigate or postpone the onset of RA slightly, but that oestrogens are not able to alleviate the symptoms (see Section 1.3.5) (Bijlsma & van den Brink, 1992).

The data from twelve conflicting studies, incorporating four different countries, was analysed, and the overall trend was for a slight protective effect in case-control studies but no effect in cohort studies (Romieu *et al.*, 1989). Another review of the literature concluded that in general, data originating from American studies failed to show a benefit from oral contraceptive use in RA, whereas European findings usually indicated considerable protection (Vandenbrouke *et al.*, 1989). When disease course was followed in RA patients over a period of 6 years in relation to past or present use of oral contraceptives, it was concluded that use prior to disease onset was associated with a reduction in the severity of the condition (van Zeben *et al.*, 1990). In their review of the literature, Spector and Hochberg (1989) also surmised that rather than being 'protective', oral contraceptives may serve to prevent the progression from mild to severe forms of RA. This may also explain the divergence between American and European studies, as the majority of European investigations were hospital based, where patients with more severe forms of the disease are likely to be included (Hazes & van Zeben, 1991). However, as any protective effect seen was independent of dose, duration of use and the presence of DR4 (Hazes *et al.*, 1990b), and the use of female sex hormones therapeutically has shown little benefit (see Section 1.3.5), this

hypothesis would also seem to be inadequate to encompass all of the complexities in this area.

1.3.4 Pregnancy

One consistent finding that links hormonal variations with changes in the immune system is that of a remission of RA during pregnancy (reviewed by Spector & DaSilva, 1992). A considerable proportion of RA sufferers have been found to go into clinical remission during pregnancy, but an equally large number suffer a postpartum exacerbation of the disease (Ostensen *et al.*, 1983; del Junco *et al.*, 1989). This pattern has been shown both in classical RA and in a patient with palindromic RA (Verwilghen & Panayi, 1992). When attempting to uncover the cause of pregnancy-induced remission in RA, levels of immune complexes and rheumatoid factors were seen to vary during and after pregnancy in a small number of patients, with some correlation between levels and the clinical changes recorded (Pope *et al.*, 1983). The levels of all the sex steroids increase dramatically during the third trimester, which could potentially act to suppress the maternal immune system. It has been proposed that progesterone is the essential hormone for control of immunity during pregnancy (reviewed by Stites & Siiteri, 1983). However, the factor(s) responsible for the postpartum flare have yet to be elucidated. When a group of RA sufferers were followed during and after pregnancy, it was found that the increased risk was highest during the first three months postpartum, but persisted for the subsequent nine months. This flare was greatest in women who had disease onset after their first pregnancy, suggesting that the hormonal changes which occur, or the exposure to paternal HLA antigens may be of influence (Silman *et al.*, 1992). Disease activity during pregnancy has also been related to the similarity between maternal and foetal HLA, with an amelioration in maternal RA associated with a disparity in HLA class II antigens between the mother and foetus, especially HLA-DRB1, -DQA and -DQB (Nelson *et al.*, 1992; Nelson *et al.*, 1993).

Rather than pregnancy *per se* influencing disease progression, it has also been suggested that lower fertility rates (del Junco *et al.*, 1989) or poor reproductive outcome (Silman *et al.*, 1988) may be risk factors for RA. However, these observations have since been disputed (McHugh, 1990; Spector & Silman, 1990). In 1992, Pritchard published a report which concluded that although pregnancy was found to ameliorate RA, it was also the most common identifiable event leading up to the onset of disease in females of childbearing age. Thus, pregnancy itself maybe a risk factor for the development of RA, perhaps explaining the protective effect seen with

oral contraceptives by some groups (see Section 1.3.3). However, it has also been reported that nulliparity is detrimental, and that nulliparous, non-oral contraceptive users had a four-fold higher risk of RA compared to parous oral contraceptive users (Spector *et al.*, 1990). The contradictions in the literature are reviewed by Hazes (1991) and Silman (1992). The influence of oral contraceptives and pregnancy is brought into context somewhat by the results of a survey into the incidence of RA in a group of nuns, and the finding that the prevalence of the disease is identical to that in the general population (McGill & Brougham, 1990).

The overall picture is that there is no benefit of pregnancy in the long-term, only whilst the immune system is compromised to prevent rejection of the foetus. Pregnancy serum was found to contain a substance which could stabilise lysosomal membranes (Hempel *et al.*, 1970) and interfere with polymorphonuclear leukocyte (PMNL) phagocytosis (Persellin & Leibfarth, 1978) and may thus account, at least in part, for the reduction in inflammation seen. This was observed in particular with third trimester serum, which correlates with the peak of disease suppression. Various factors released during pregnancy have the potential to modulate the inflammatory process in such a way that they have an ameliorating effect in RA (reviewed by Nicholas & Panayi, 1988). Such factors include alphafetoprotein (Lu *et al.*, 1984) which can inhibit macrophage class II expression, and pregnancy-associated α_2 glycoprotein which has been reported to reduce human monocyte Fc receptor and HLA-DR expression (Thomson *et al.*, 1979; Persellin & Rhodes, 1981). However, the immunomodulatory activities of certain factors are now thought to be due to contaminating species, for example HCG, which was found to be contaminated with uromodulin, a naturally occurring IL-1 inhibitor (see Section 1.8.1) (Muchmore & Decker, 1985). When pregnancy serum was fractionated, two regions of immunosuppressive activity were located and it was postulated that these exist in normal sera as inactive complexes and are activated during pregnancy (Davies & Browne, 1985).

1.3.5 Therapeutic Uses of Sex Hormones

The first female sex hormone preparations became available in the early 1960s and their effect on RA was subsequently investigated. Norethynodrel, a combination of ethinyloestradiol and progesterone resulted in some improvement in all of the six patients initially studied (Blais & Demers, 1962). More recently, when female patients were treated with ethinyloestradiol alone a certain degree of amelioration of symptoms was recorded (van den Brink *et al.*, 1989), whereas with patients treated with the high-

dose oral contraceptive *Lyndiol* no effect was reported (Hazes *et al.*, 1989c). The use of hormone replacement therapy in RA has also been found to be ineffective (Carette *et al.*, 1989), whereas the treatment of peri- and postmenopausal women with non-contraceptive hormones prior to disease onset considerably reduced the risk of subsequent development of RA (Vandenbrouke *et al.*, 1986). However, a recent review concluded that the use of oestrogens in RA is an ineffective form of therapy (Bijlsma & van den Brink, 1992). Progesterone has also been used, as an intra-articular administration in twelve RA patients, with potent local anti-inflammatory effects, possibly as a result of agonistic glucocorticoid-like effects at high steroid levels (Cuchacovich *et al.*, 1988).

The testosterone deficiency recorded in a high proportion of male RA sufferers lead to the treatment of seven male patients with 120 mg/day testosterone undecanoate for six months (Cutolo *et al.*, 1991). This resulted in an increase in total testosterone and DHEAS levels and a reduction in FSH and LH, which was associated with improvements in disease parameters and clinical status, including an increase in the number of CD8+ T cells and a reduction in the CD4:CD8 ratio (see Section 1.6.2). Therefore, although some success has been recorded with the use of oestrogens and androgens as therapy in RA further investigations are required before any firm conclusions can be made. All the hormones used to date have been synthetic and it may prove worthwhile to use naturally occurring hormones in the future.

1.4 THE INFLUENCE OF SEX HORMONES IN SYSTEMIC LUPUS ERYTHEMATOSUS

Whereas in RA females seem most susceptible to development of the disease at the time of the menopause, females of child-bearing age are most susceptible to SLE. In this age-group the female:male ratio is 9:1. However, the ratio is considerably reduced in prepubertal children and after the menopause (reviewed by Lahita, 1985). There is also a link with Klinefelter's syndrome, in that increased oestrogenicity in males with the disorder leads to an heightened tendency to develop autoimmune conditions, including SLE and RA (Stern *et al.*, 1977). Pregnancy has been associated with both the onset of the disease (Mund *et al.*, 1963) and increases in the severity in patients with disease onset prior to pregnancy (Tincani *et al.*, 1991). In addition, fluctuations in endogenous hormonal levels during the normal menses have also been stated to have detrimental effects on disease activity, with approximately 60% of patients in one study reporting adverse symptoms in the ten days prior to menstruation (Rose & Pillsbury, 1944). As

with RA, the use of oral contraceptives is a rather confounding issue. A worsening of disease symptoms was reported with oestrogen oral contraceptives, whereas pure progesterones did not result in an exacerbation (Jungers *et al.*, 1982). However, in a later study of eighty-five female SLE patients no such association was found, although most of the patients included suffered only mild disease, in contrast to the Jungers study which consisted solely of those with severe SLE (Julkunen, 1991).

1.4.1 Abnormalities in Sex Hormone Metabolism

SLE has been associated with an increased 16α-hydroxylation of oestrone resulting in elevated levels of metabolites which are highly oestrogenic. This was found to occur in both male and female patients, and family data indicated that it could be an inherent abnormality (Lahita *et al.*, 1982). Steroids such as cortisol and 16α-hydroxyoestrone which possess a stable ketol moiety are able to form stable covalent adducts with proteins. The reaction occurs preferentially with membrane proteins and increased levels of these 16α-hydroxyoestrone-protein adducts have been found on the membranes of red cells and lymphocytes of female SLE patients and also in pregnant women (Bucala *et al.*, 1984; Bucala *et al.*, 1985). Anti-oestrogen antibodies were found to correlate with levels of plasma 16α-hydroxyoestrone and with active disease which may contribute to the pathogenicity of SLE (Bucala *et al.*, 1987; Counihan *et al.*, 1991). Interestingly, antibodies to the 16α-hydroxyoestrone-modified proteins were detected in 26% of SLE patients and in 25% of normal, disease-free females with a history of oral contraceptive use (Bucala *et al.*, 1987; Counihan *et al.*, 1991).

An increased oxidation of testosterone at the C-17 position has been reported in females with active disease, which would also have the potential to enhance levels of the female sex hormones (Lahita *et al.*, 1983). Hormonal variations due to increased 16α-hydroxylation and oxidation at C-17 have been postulated to be important in the enhanced disease symptoms seen during pregnancy (reviewed by Lahita, 1992). In males with SLE, increased resting serum levels of oestrone, androstenedione and LH were detected, with concomitant reductions in testosterone, DHT and oestradiol (Lavalle *et al.*, 1987). It was concluded that androstenedione to testosterone, and testosterone to DHT conversions were impaired, with the resulting low testosterone and DHT in turn stimulating LH release via the normal feedback mechanism. This supports the work of Lahita *et al.* (1982) showing the preferential accumulation of 16α-hydroxylation products, androstenedione and oestrone. Reduced serum oestradiol and increased testosterone have also been reported in a group of postmenopausal SLE

patients (Folomeev *et al.*, 1989). Other abnormalities in hormonal metabolism in SLE include progesterone deficiency (Arnalich *et al.*, 1992) and elevated prolactin levels (Lavalle *et al.*, 1987). However, the general finding in SLE is of an abnormality in sex hormone metabolism resulting in a reduction in androgen levels and a hyperoestrogenic state (reviewed by Talal & Ansar Ahmed, 1987).

1.4.2 Therapeutic Uses of Sex Hormones

As a result of studies into abnormal hormonal metabolism in SLE, compounds with potent antioestrogenic and proandrogenic effects were considered as potential therapeutic agents. Androgens and synthetic hydroxy progesterone derivatives have been tried with variable results (reviewed by Asherson & Lahita, 1991). In five patients with Klinefelter's syndrome associated with autoimmune disease, three with Sjogren's syndrome and two with SLE, treatment with testosterone undecanoate resulted in a correction of abnormal testosterone and LH levels, as well as normalisation of CD3+ and CD8+ numbers, with a concomitant correction of the CD4:CD8 ratio (Bizzarro *et al.*, 1987). In addition, all patients treated went into a clinical remission.

1.5 ANIMAL MODELS OF AUTOIMMUNITY

The link between gender, sex hormones and autoimmune disease is more clearly seen in animal models (reviewed by Ansar Ahmed *et al.*, 1985a; Lahita, 1985; Holmdahl *et al.*, 1989; Ansar Ahmed & Talal, 1990). Type II collagen-induced arthritis (CIA), a common experimental model for arthritis in rats and mice, appears to be controlled by sex hormones. It has been shown, for example, that sex hormone-related phenomena such as pregnancy-induced remission and postpartum flare of the disease, can be reproduced in this animal model (Mattson *et al.*, 1991). Treatment of mice with 17β-oestradiol delayed the onset without affecting the incidence of CIA, whereas treatment after the onset reduced both the severity and duration of the disease (reviewed by Holmdahl *et al.*, 1989).

The F1 hybrid mouse strain from B10Q and DBA/1 parentals (QD strain) is highly susceptible to CIA, with the incidence being greater in males than females, in contrast to human RA. Treatment with oestrogen resulted in a reduction in disease manifestations in both castrated and non-castrated males, whereas testosterone implants in normal female mice caused an exaggeration of CIA, via an inhibition of ovarian

oestrogen production (Jansson & Holmdahl, 1992). Collagen-induced T cell-mediated arthritis in DBA/1 mice is also readily inducible in males rather than females, and the male preponderance for development of CIA has been demonstrated in other mouse strains, with oophorectomy significantly enhancing the expression of the disease in females (Holmdahl *et al.*, 1986). In contrast to the above mouse models, in Lewis rats CIA is readily induced in females rather than males. However, castration of female rats produced similar effects to castration of female mice, in that an increase the incidence of polyarthritis occurred, which was reversed following treatment with 17β-oestradiol (Larsson & Holmdahl, 1987), further supporting an important role for oestrogen in the protection against this model of RA.

The above results with CIA demonstrate an apparent role for oestrogen in the protection against the development and/or reduction in severity of the disease. However, in other animal models of autoimmunity androgens appear to be the significant contributory factor. For example, male LEW/N rats are relatively resistant to an induced form of polyarthritis, thought to be due to the presence of male sex hormones, with orchidectomy or oestrogen therapy increasing their susceptibility (Allen *et al.*, 1983). MRL-lpr mice, which spontaneously develop an aggressive lupus and lymphoproliferative disorder, show reduced antibody levels following treatment with androgens (Steinberg *et al.*, 1980). Thus, oestrogens and androgens can have opposing effects on different autoimmune conditions, and potentially on the same autoimmune condition expressed in different animal models.

Perhaps the most widely reported animal model of autoimmunity is the New Zealand black (NZB)/New Zealand white (NZW) F1 hybrid mouse model (B/W). Females have an accelerated expression of lupus and Sjogren's syndrome-like diseases, compared with males, with high levels of circulating antibodies and immune complexes. This strain is considered a laboratory model for SLE and demonstrates the opposing hormonal effects to those seen with animal models of arthritis, in as much as oestrogens exaggerate and androgens ameliorate the condition. Prepubertal orchidectomy increased expression of the disease in males and replacement with the male sex hormone DHT delayed the onset (Roubinian *et al.*, 1978). Prepubertal castration of females, in contrast, had no effect on disease progression. However, when castration of female or male mice was combined with 17β-oestradiol alone, or in conjunction with either DHT or progesterone, increased mortality, autoantibody production and immune complex nephritis was observed (Roubinian *et al.*, 1979a). The increase in mortality in males was reduced following administration of progesterone alone, whereas danazol, a weakly androgenic preparation, was ineffective, but the anti-

androgen, cyproterone acetate, lead to premature autoantibody production in male mice. DHT also increased survival of female B/W mice, even if given after the onset of disease, although anti-DNA antibodies were still found to be present, suggesting that the mode of action may be via a reduction in immune complex deposition (Roubinian *et al.*, 1979b). In addition, androgens reduced and oestrogens enhanced spontaneously- and immunisation-induced antibodies to ssDNA in NZB/NZB, NZB/C3H, NZB/CBA and NZB/DBA mice (Steinberg *et al.*, 1979). Young castrated male B/W mice with an oestrogen implant produced increased IgM antibodies to a variety of antigens, whereas testosterone was ineffective. Older female B/W mice receiving testosterone responded in the same manner as age-matched males (Brick *et al.*, 1985).

Evidence points to a direct effect of sex hormones on immunity. However, it is also possible that differences in immune reactivity between the sexes is due to an effect of sex chromosomes, or genetic background, rather than a direct hormonal effect. For example, NZB mice appear to be insensitive to the suppressive effects of testosterone, an effect which may be genetically mediated (Raveche *et al.*, 1979). In addition, not all mouse strains are susceptible to oestrogen-mediated suppression of CIA, therefore, there may be a genetic restriction to sensitivity (reviewed by Holmdahl *et al.*, 1989). Holmdahl *et al.* (1992) reported that the spontaneously developing arthritis in DBA/1 mice is dependent on DBA/1 recessive genes. When F1 hybrids were created between this strain and BXSB mice, earlier development of the disease was seen, suggesting that the BXSB genetic background had a permissive and contributory effect on disease progression. In addition, there is some evidence for a Y-chromosomal link with the susceptibility of male BXSB mice to develop a lupus-like syndrome (Murphy & Roths, 1979). X-linked genes may also contribute to differences in immune function observed, for example, experimental autoimmune thyroiditis in rats was thought to be associated with the X chromosome (Lillehoj *et al.*, 1981). To overcome this problem either reciprocal crosses or castrated and hormonally altered animals were studied, with the results indicating that although genetic influences may come into play, sex hormones *per se* seem to have an important role in controlling the immune system (Steinberg *et al.*, 1979).

Other problems encountered when studying hormonal effects *in vivo* include, the ability of testosterone to be metabolised to oestrogen, the dose of hormones used, the age of administration of the hormones and the differential effects on differing autoantibodies and various immune responses studied (Steinberg *et al.*, 1979). Behaviour can also seemingly affect disease outcome (Holmdahl *et al.*, 1992). Therefore, although it is

possible to overcome these considerations via careful experimental design, it is important to keep an open mind when interpreting the literature.

In summary, oestrogen has been shown to enhance autoantibody production in certain autoimmune conditions which rely on B cell hyperactivity, such as SLE, but oestrogens can also seemingly reduce autoimmunity mediated by T cells, for example in RA. However, this is likely to be a gross oversimplification of a complex balance of the hormones and their metabolites occurring *in vivo*.

1.6 SEX HORMONES AND THE IMMUNE SYSTEM

In 1968 Terres *et al.* reported a quantitative difference between the immune response of males and females. The observations were made when studying antibody production in Swiss Albino mice following an antigenic challenge, when it was found that female mice produced 10-85 times more antibody than males. Previous observations of qualitatively higher immunoglobulin levels in normal female adults and children, of both IgM (Butterworth *et al.*, 1967) and IgG (Lichtman *et al.*, 1967) subtypes had been made. In general, it seems reasonable to conclude that females have greater immunity, both humoral and cell-mediated, than males, although there has been some disagreement on this, with Santoli *et al.* (1976) stating that, in humans, males have the stronger cell-mediated immune response.

When considering individual hormones, oestrogens, androgens and progesterone all seem able to modulate the cell-mediated immune system. The following Sections concentrate mainly on the effects of oestrogens and androgens, although progesterone has also been reported to have important immunosuppressive actions such as increasing skin graft survival (Kincl & Ciaccio, 1980), reducing immune spleen cell function (Sekiya *et al.*, 1975) and enhancing suppressor T cell activity *in vitro* (Holdstock *et al.*, 1982). The effects of progesterone and other sex hormones on immune function and autoimmunity have been widely reviewed (Stites & Siiteri, 1983; Grossman, 1984; Ansar Ahmed *et al.*, 1985a; Lahita, 1985; Talal & Ansar Ahmed, 1987; Ansar Ahmed & Talal, 1990; Lahita, 1990; Denman, 1991). Many of the initial studies of sex hormone effects in the immune system related to modulation of thymus function. More recently, specific immune cell types have been analysed.

1.6.1 The Thymus

Administration of sex hormones *in vivo* was found to result in a change in thymic weight, with oestrogenic compounds causing hypertrophy and androgenic compounds causing atrophy (Chiodi, 1940). These findings, along with the observation that both oestradiol (Grossman *et al.*, 1982) and testosterone (Grossman *et al.*, 1983) were able to regulate the release of thymic serum factors, fuelled ideas that hormonal modulation of the immune system may occur via the thymus. Grossman *et al.* (1982; 1983) performed experiments to study the *in vitro* response of thymic lymphocytes to concanavalin-A (ConA) or phytohaemagglutinin (PHA) under the influence of specific thymic factors from either control, castrate, thymectomised or castrate and thymectomised male rats, treated with or without oestradiol or DHT. They reported that in cultures with castrate rat serum there was an increased response to both mitogens which could be reduced by using sera from oestrogen-treated animals. A heightened response to PHA was also seen with sera from thymectomised rats and this was lost when the animals had been treated with either oestradiol or DHT. No stimulation of the PHA response was seen in castrate, thymectomised animals. This led to the hypothesis that there are thymic-derived serum stimulatory and inhibitory factors, and gonadal stimulatory and inhibitory factors, with the latter affecting the former, and with all under the influence of the sex hormones.

There have also been a number of *in vivo* studies directed towards finding specific thymic-derived factor(s) which are controlled by sex hormones and which, in turn, affect immunity. In 1991, Erbach & Bahr measured oestrogen effects on antibody responses in ovariectomised adult Lewis rats which had been thymectomised or sham-operated. It was found that an intact thymus and oestradiol treatment were prerequisites for enhanced antibody production in response to a given stimulus, a response which could be mimicked in ovariectomised/thymectomised animals by the administration of oestradiol and thymosin fraction 5. Thymic epithelial cells produce a number of soluble factors and hormones which are involved in the development of the T cell system and hence in the regulation of immunity. Many of the activities were found to reside in thymosin fraction 5, which is a semipurified aqueous extract of calf thymus. Thus, it appears that potentiation of humoral immunity by oestrogen requires a constitutive thymic factor. As injection of immunodeficient old mice with synthetic thymosin α_1, a 28 amino acid residue peptide from thymosin extract 5, restored T-helper cell activity, IL-2 production and IL-2 receptor expression (Doria *et al.*, 1992) it is tempting to speculate that such a factor could be crucial in the development and maintenance of a functional immune system.

The first reports of sex hormone receptors within the immune system were of oestrogen and androgen binding sites in the thymus (reviewed by Grossman, 1984). These observations were made using rat and mouse tissue and there was some contention as to whether the receptors were localised to the reticuloepithelial matrix of the thymus, or whether their distribution was more widespread. Recently, androgen receptors (AR) have been found on human thymocytes (Kovacs & Olsen, 1987), indicating that sex hormone receptors have various locations within the thymus. In support of a predominantly reticuloepithelial location for sex hormone receptors, Luster *et al.* (1984) found that it was the supernatants from thymic epithelial cultures, rather than from thymocytes, when incubated with oestrogen, that were compromised in terms of supporting mitogenic responses. However, the proceding observations provide overwhelming evidence for the additional presence of receptors on thymocytes.

The enzyme 20αhydroxysteroid dehydrogenase (20αSDH) is located in thymocytes, θ-bearing splenic lymphocytes and in bone marrow pre-T-lymphocytes (large cells), but not in non-lymphoid haematopoietic cells, and is therefore used as a marker for thymic, as well as splenic and bone marrow cells. By measuring changes in 20αSDH activity it was found that when testosterone and its metabolites were administered to castrated mice there was an increase in the number of large cells in the bone marrow, with a concomitant reduction in thymic cell number. However, the residual thymic cells were of the more mature type, responded vigorously to PHA and ConA and possessed most of the 20αSDH activity (Weinstein & Berkovich, 1981; Weinstein & Isakov, 1983). It was suggested that changes caused by testosterone in the marrow affected the thymus via alteration of thymic cell subpopulations (Weinstein & Berkovich, 1981). In addition, oestrogen has been found to affect thymic cell populations and, as with testosterone, it was found that oestradiol implants in castrated B/W mice led to an increase in mature thymocytes. These were of the 'helper' rather than 'suppressor' phenotype, ie. those with Lyt 1+2- antigens on their cell surface (Novotny *et al.*, 1983). It was postulated that the effects of oestradiol on the thymus were both direct and indirect, with direct action on thymic ER, and indirectly via an alteration in bone marrow precursor cells. More recently oestrogen effects on a broad range of thymocyte subpopulations have been studied (Screpanti *et al.*, 1991). The major consequence of oestradiol administration to mice was seen to be a redistribution of subsets which appeared to occur via a selective depletion of 'immature' and 'intermediate' thymocyte cell populations. The enrichment of 'mature' cell subsets which resulted could have been due to the loss of the less mature subsets, or could theoretically have been due to oestrogen driving cells towards further steps of differentiation. As IL-1α mRNA levels were elevated in the thymus following oestrogen exposure, and IL-1 has also been

shown to alter thymocyte subset patterns (Fowlkes *et al.*, 1987), it was hypothesised that oestrogen may be acting by stimulating the release of this important cytokine.

1.6.2 T Cells

Sex hormones have been reported to influence T cell responses both *in vitro* and *in vivo*. Various hormones, including oestradiol, progesterone, testosterone, diethylstilbestrol (DES), cortisol and 11-desoxycortisol (a steroid with low biological potency) were all able to inhibit PHA- and ConA-induced human lymphocyte proliferation, whether the donor was male or female (Ablin *et al.*, 1974; Mendelsohn *et al.*, 1977; Wyle & Kent, 1977). Other observations such as increased tumour survival in oestrogen-treated female mice compared to androgen-treated females, contrasting to increased tumour survival rate in androgen-treated male mice, demonstrate that oestrogens and androgens are able to act preferentially on female and male cells, respectively (Franks, 1975). When responses of human female lymphocytes to PHA-stimulation were studied during the menstrual, follicular and luteal phases of the menstrual cycle, no difference was seen (Caggiula *et al.*, 1990), indicating that normal endogenous fluctuations in hormone levels do not alter this one aspect of immune function.

Weinstein *et al.* (1984) looked at lymphocyte responses in various strains of mice and found cells from female or testosterone-insensitive males to be more reactive in the mixed lymphocyte reaction (MLR). Oestrogen implants in male mice led to an increased responsiveness. In addition, castration of male mice enhanced and androgen treatment of females depressed antigen presentation, once again demonstrating the opposing effects of male and female sex hormones in immunity. In the MLR in rats, tamoxifen was seen to be inhibitory, whereas oestrogen was ineffective at physiological concentrations but reduced reactivity at pharmacological concentrations (Baral *et al.*, 1991). In a study of oestrogen and testosterone modulation of DTH and antibody production in a variety of normal and autoimmune mouse strains, Carlsten *et al.* (1989) concluded that the effects of both sex hormones on immune functions are genetically linked but that the genetic basis for hormone susceptibility is different for oestrogen and testosterone. It has since been suggested that the gene encoding for oestrogen-mediated suppression of DTH is specific for T cell-dependent inflammatory responses and is independent from histocompatibility products (Carlsten & Tarkowski, 1993).

The finding that the MLR could be enhanced in both sexes by removing suppressor T cells (CD8+ T cells) or by the addition of a T cell growth factor (Weinstein *et al.*, 1984), led to the hypothesis that oestrogen may selectively act on this T cell population. This observation was further supported by the findings of studies with normal and autoimmune mice, whereby oestrogen depressed the Lyt-2+ cell population (analogous to human CD8+ T cells) and testosterone enhanced or maintained levels (Ansar Ahmed *et al.*, 1985b). Binding sites for dexamethasone, oestrogen, progesterone and androgens were detected on human leukaemic cells (Danel *et al.*, 1981) and ER was shown to be present on human PBMC (Danel *et al.*, 1983). Subsequently, Cohen *et al.* (1983) further purified T cells from thoracic duct lymph and found that ER were present on the CD8+ subpopulation, with AR being undetectable. Kovacs & Olsen (1987) also failed to demonstrate AR on human PBMC, T cells or Jurkat cells. Stimson (1988) provided further evidence for the presence of ER on the CD8+ T cell population, and found the CD4+ subset to be ER-negative. In addition, a CD8+-enriched spleen cell population responded to 17β-oestradiol with a dose-dependent increase in immunoglobulin secretion, primarily IgM, whereas CD4+-enriched spleen cells were unresponsive (Stimson, 1988). The theory thus proposed was that oestrogen acts to depress the CD8+ population of T cells, leading to an increase in B cell differentiation and an increased antibody production.

CD4 and CD8 molecules are members of the immunoglobulin family of adhesion molecules (see Section 1.7). CD8 acts as a co-receptor with the T cell receptor for class I MHC molecules on the surface of suppressor/cytotoxic T cells. CD4 is a co-receptor with the T cell receptor for class II MHC on helper/inducer T cells. Thus, both molecules are essential for an effective immune system (reviewed by Springer, 1990). Veys *et al.* (1982) reported a reduction in CD8+ T lymphocytes in the peripheral blood of RA patients, whilst CD3+ and CD4+ cell numbers remained unchanged, hence the CD4:CD8 ratio was seen to increase. However, in paired synovial fluid samples, CD4+ cells were detected at lower levels and CD8+ cells were increased compared to peripheral blood. Emery *et al.* (1987) utilised a different panel of monoclonal antibodies (mAb) which allowed a distinction to be made between helper/inducer (CD4+4B4+) and suppressor/inducer (CD4+2H4+) cell populations, the latter of which induce CD8+ cells. They reported a depletion of the CD4+2H4+ subset in blood and synovial fluid samples from RA patients, and an increase in the ratio of the two subsets, especially in synovial fluid. However, no difference in the CD4:CD8 ratios could be found. The group concluded that the reduced CD4:CD8 ratio reported in RA synovial fluid could be due to an underlying deficit in the CD4+2H4+ subset and that, when the balance

between the two CD4+ subsets becomes significantly altered, failure to induce CD8+ cells may occur.

When studying CIA in rats, an anti-arthritic effect with oestrogen was seen in the absence of CD8+ T cells (Larsson *et al.*, 1989). However, depletion of CD8+ T cells itself reduced the incidence of CIA in non-oestrogen treated, thymectomised rats, suggesting that this cell subset is of importance. Rather than a direct effect of oestrogen on CD8+ cells, an increase in the murine T-helper cell (CD4+) subset has also been reported with oestrogen (Novotny *et al.*, 1983), and in ten patients treated with a daily dose of a CD4 mAb, six showed a clinical response (Reiter *et al.*, 1991).

In premature ovarian failure T lymphocyte activation was enhanced, in terms of higher HLA-DR expression and elevated IL-2 receptor expression (Nelson, *et al.*, 1991). In this condition, the relative number of CD4+ and CD8+ counts increase, but there is a reduction in the CD4:CD8 ratio (Pun & Ho, 1990), thought to be due to oestrogen deficiency (Ho, 1992). Therefore, it may be the ratio of T cell subsets which is important, rather than effects on individual cell types, and it has been postulated that cyclical changes in oestrogen release by the ovaries stimulates the immune system through changes in the CD4:CD8 ratio, leading to the heightened susceptibility to autoimmunity in females. However, as previously mentioned, the response of female lymphocytes to PHA was unaltered by the stage of menstrual cycle (Caggiula *et al.*, 1990). Although much of the literature points to a unique role for oestrogen in modulating T cell activity, Weinstein & Berkovich (1981) demonstrated that castration decreased, and testosterone-treatment increased, suppressor T cell activity in mice, and proposed that testosterone may also be an important contributory factor in the female prevalence in autoimmune disease.

1.6.3 B Cells

Physiological concentrations of oestrogen were found to stimulate, and testosterone inhibit, pokeweed mitogen (PWM)-induced B cell differentiation *in vitro*, using peripheral blood lymphocytes from various donors (Paavonen, 1981; Sthoeger *et al.*, 1988; Kalman *et al.*, 1989). High concentrations of oestradiol were inhibitory (Paavonen, 1981). The effect was measured as an increase in IgG rather than IgM antibodies, and there was no difference between males and pre- or postmenopausal females (Weetman *et al.*, 1981; Sthoeger *et al.*, 1988). Antibody formation in response to T cell-dependent and -independent antigens in rats was increased by oestrogen and,

in contrast to findings with human lymphocytes, this was thought to be due to a potentiation of B cell IgM production (Myers & Petersen, 1985; Dahlgren & Hanson, 1991). Oestrogen was also seen to increase the percentage of CD5+ B cells, correlating with a rise in autoimmunity in both normal and autoimmune-prone mice, in terms of increased antibodies against bromelain-treated mouse red blood cells (Ansar Ahmed *et al.*, 1986).

When various mice strains were orchidectomised and treated with testosterone, there was no change in autologous plaque-forming cells, whereas those receiving oestrogen had an increase in autoantibodies. This was thought to be due to a direct effect on CD5+ B cells, in that oestrogen enhanced the antibody forming capacity of this subpopulation (Ansar Ahmed *et al.*, 1989; Talal *et al.*, 1992). CD5+ B cells are increased in Sjogren's syndrome and RA, but not in SLE, and are thought to be linked to immunoregulation. Indeed, CD5+ B cells have been shown to produce autoantibodies in autoimmune mice, and in patients. In RA it is thought that they may be under genetic control or serve as an activation marker, as levels are highest in the foetus and generally decline during development, but remain elevated in autoimmunity. It is thought that their role may include that of first-line defence or general removal of cell debris, or that of regulator of B and T cell functions, perhaps by amplifying the immune system (reviewed by Plater-Zyberk *et al.*, 1992). Thus, it is possible that B cells as well as T cells are an important target for hormonal modulation in autoimmunity.

1.6.4 NK Cells

CD3- large granular lymphocytes, or natural killer (NK) cells, found mainly in the spleen and blood, provide crucial defence against viral infections. Various groups have reported a reduction in NK activity following oestrogen treatment *in vivo*, in both normal and autoimmune models, although high doses of oestrogen were often used (Seaman & Gindhart, 1979; Ansar Ahmed *et al.*, 1986). This was thought to depend on the bone marrow because it became evident when osteoproliferation caused a reduction in bone marrow capacity, also a result of oestrogen therapy. In B/W mice, diminished NK levels correlated with an amelioration of disease symptoms, suggesting that this cell type may also be important in autoimmunity (Seaman & Gindhart, 1979).

1.6.5 Monocytes/Macrophages

Baranao *et al.* (1991) studied peritoneal macrophages from BALB/c mice and found that following castration of female and male animals a reduction in the numbers of functionally active complement (C3b) receptors and a suppressed phagocytic response occurred. Oestrogen treatment of ovariectomised mice restored phagocytosis to normal levels and treatment with progesterone re-established C3b receptors. DHT had no effect, which supports data showing high-affinity binding sites for oestrogen in a human monocytic cell line and rat peritoneal macrophages, with receptors for DHT being undetectable (Gulshan *et al.*, 1990). Both oestrogens and progesterones have also been shown to increase class II expression and IL-1 production by mouse peritoneal macrophages (Flynn, 1986).

1.6.6 Neutrophils

The oxidative metabolism of polymorphonuclear leukocytes (PMNL) was found to be increased *in vitro* when using micromolar concentrations of the oestrogenic hormones, β-oestradiol, oestrone, 16α-hydroxyoestrone and oestriol. All were thought to act via an increase in myeloperoxidase (Jansson, 1991). The corresponding 2-OH-compounds were inhibitory in this system, therefore it may be the ratio of the different oestrogenic metabolites which is important in controlling neutrophil oxidative metabolism. Male sex hormones were inactive. In contrast, Buyon *et al.* (1984) found that pharmacological levels of oestrogen inhibited neutrophil superoxide production and degranulation.

1.7 SEX HORMONES AND CELL ADHESION

The circulation of immune cells around the body and their passage through tissues is an essential part of natural surveillance against infections (reviewed by Springer, 1990). This requires rapid transition between non-adherent and adherent states, and *vice versa.* The adhesion molecules are becoming increasingly popular as candidates for many such immune interactions and have been proposed as possible inflammatory mediators (reviewed by Panayi, 1993).

Three families of adhesion molecules are responsible for mediating an immune response. The immunoglobulin superfamily includes the antigen-specific T and B cell receptors and the CD4 and CD8 molecules, the role of which has been discussed (see

Section 1.6.2). The lymphocyte function-related antigens, LFA-2 (CD2) and its counter-receptor LFA-3, essential for T cell activity, are also members of the immunoglobulin family. Selectins are involved in lymphocyte and neutrophil interactions with the vascular endothelium and the integrin family are important in the dynamic regulation of adhesion and migration (reviewed by Springer, 1990). The three families are by no means exclusive, indeed there is considerable overlap in terms of receptors and their ligands. This section concentrates on the integrins as they appear to play a critical role in the pathogenesis of RA in terms of the infiltration and perpetuation of cells within the joint.

The integrins are a superfamily of heterodimers with non-covalently associated α- and β-subunits which are responsible for mediating cell-cell and cell-matrix interactions (reviewed by Hogg, 1991; Hynes, 1992). Recently the family has been subdivided into groups according to the β-subunit with which the α-subunits associate: the VLA proteins which share a common β1-subunit; the leukocyte integrin (CD11/CD18) family sharing a common β2-subunit, and the cytoadhesins, or β3 type. However, this is somewhat of an oversimplification as a total of eight β-subunits have now been described, with a total of fourteen α-subunits known to date. In addition, certain α-subunits, for example αV, can form multiple β-pairings, such as αVβ1, αVβ3 and αVβ5, all of which have different functional roles. It seems that both the α- and β-subunits are responsible for the formation of the ligand-binding site. Integrin function is either Mg^{2+}- or Ca^{2+}-dependent due to a divalent cation binding site.

The leukocyte integrins include LFA-1 (CD11a/CD18 or αLβ2), CR3 or Mac-1 (CD11b/CD18 or αMβ2) and p150,95 (CD11c/CD18 or αXβ2). CR3 and p150,95 are generally confined to cells of the myeloid lineage and perform functions such as monocyte adherence to endothelium, chemotaxis and phagocytosis. LFA-1 is present on monocytes and T cells and contributes to the interaction between the two cell types. LFA-1 binds ligands ICAM-1 and ICAM-2, which are integral membrane proteins of the immunoglobulin superfamily. Other such ligands which can mediate direct cell-cell adhesion are ELAM-1 and VCAM-1. ELAM-1 is a member of the selectin family and is often termed E-selectin, whereas VCAM-1 belongs to the immunoglobulin adhesion molecule family. The leukocyte integrins are essential for interactions in an immune response, both between cells and with the extracellular matrix (reviewed by Dransfield, 1991).

The VLA proteins were first identified on T cells in long-term culture, hence the term 'very late activation' (VLA). They are now known to be expressed on a wide range of

different cell types. The function of the VLA integrins is mainly one of cell-matrix interactions with collagen, laminin and fibronectin, but they have recently been demonstrated to participate in cell-cell interactions. Low levels of VLA-3 and higher levels of VLA-4, VLA-5 and VLA-6 are expressed on resting and memory T cells. VLA-1 and VLA-2 are detectable only on activated T cells, and B cells express a different array of these integrins (reviewed by Shimizu & Shaw, 1991). The cytoadhesins are also found on leukocytes. The receptor GPIIb/IIIa (CD41/CD61) is limited to platelets and megakaryocytes, and functions in the clotting cascade, whereas, the vitronectin receptor (CD51/CD61) is expressed on a wider range of cell types, including tissue culture-matured monocytes, and has recently been shown to engage in cell-cell interactions.

Cellular activation is often required for the expression of certain integrins or for ligand-binding to occur, and different stages of activation may be necessary to result in a stable interaction, with the stimuli dependent on the cell type (reviewed by Dransfield, 1991; Hogg, 1991; Hynes, 1992). For example, the VLA binding activity of T cells is rapidly and dramatically increased following activation, but without any change in the surface expression of these molecules (Shimizu *et al.*, 1990). In addition, costimulation can occur between ligand receptors, and it is possible that engagement of an integrin receptor can trigger a signal back into the cell to modulate the expression or production of some other factor. It is now thought that the 'clustering' of integrins on the cell surface can activate tyrosine kinase leading to phosphorylation and signal transduction (reviewed by Kornberg & Juliano, 1992).

Duke *et al.* (1982), found that CD4+ T cells were the predominant lymphocyte population infiltrating the synovial membrane of affected joints in RA, and it was postulated that, during active phases of the disease, it is the 'memory' T cell subpopulation of the CD4 subset (CD45RO+) which is increased (Pitzalis *et al.*, 1991). Enhanced adhesiveness of the CD4+CD45RO+ T cells is one possible explanation for their preferential accumulation within the synovial membrane. Indeed, it has been shown that the memory phenotype is associated with binding to ELAM-1 (Shimizu *et al.*, 1991). Synovial T cells have been shown to display an enhanced capacity to interact with ELAM-1 and VCAM-1 (Postigo *et al.*, 1992), and increased levels of circulating ICAM-1 and VCAM-1 have been recorded in RA patients, especially in the synovial fluid (Mason *et al.*, 1993). This could occur either by prior activation of the T cells and/or by activation of the endothelial cells, causing an upregulation of adhesion molecules and hence potentiating the interaction between the two cell types. The release of cytokines at an inflammatory site has the potential to mediate such interactions

(reviewed by Pober & Cotran, 1990). In this respect, IL-1 and TNF have been shown to increase the expression of ELAM-1, ICAM-1 and VCAM-1 on endothelial cells, and both ICAM-1 and VCAM-1 play an important role in binding T cells to resting or IL-1-activated endothelium, respectively (Oppenheimer-Marks *et al.*, 1991). In addition, IFN-γ increases adhesion for lymphocytes specifically, and can synergise with TNF in the induction of ICAM-1 expression (reviewed by Pober & Cotran, 1990).

The transition of monocytes from the general circulation to an inflammatory site is also thought to be mediated by adhesion molecules. VLA-4 and all three members of the β2 (CD18) leukocyte integrin family are amongst those expressed on the monocyte cell surface (reviewed by Carlos & Harlan, 1990). It has been shown that, apart from a general upregulation of these molecules and increased binding of VLA-4 to VCAM-1, for example, the selectins, in particular P-selectin, are important in monocyte-endothelial interactions in RA (Grober *et al.*, 1993). As with other cell types, cytokines have the potential to modulate adhesion molecule expression on monocytes . Short-term incubation of whole blood with IL-2, interleukin-4 (IL-4), TNF-α or TNF-β altered the expression of monocyte CD18 and/or CD11a, b and c expression to various extents, whereas IL-1β, interleukin-6 (IL-6) and IFN-γ were ineffective (Limb *et al.*, 1992). In addition, IFN-γ, TNF-α, IL-1 and granulocyte-macrophage colony-stimulating factor (GM-CSF) all increased ICAM-1 expression on peripheral blood monocytes (Diaz-Gonzalez & Alvaro-Gracia, 1992). Lymphocytes require a longer activation period than monocytes to observe changes in expression of such molecules.

Subsequent to the adhesion to endothelial cells, migration of lymphocytes and monocytes through the endothelium and tissue occurs. It is ICAM-1 rather than VCAM-1 which seems to play a crucial role in this respect, hence LFA-1/ICAM-1 interactions are important (Oppenheimer-Marks *et al.*, 1991).The perpetuation of cells within the joint is likely to involve both cell-cell interactions and also binding to the extracellular matrix, via the VLA integrins and other non-integrin adhesion molecules (reviewed by Shimizu & Shaw, 1991). Synovial fluid T cells (Garcia-Vicuna *et al.*, 1992; Rodriguez *et al.*, 1992) and macrophages (Diaz-Gonzalez & Alvaro-Gracia, 1992) have been shown to have an upregulation of certain VLA subunits and an increased binding to fibronectin when compared to peripheral blood cells, indicative of an increased state of activation. The adherence of cells of the immune system to fibroblasts has also been reported to be modulated by cytokines (Piela & Korn, 1988; Krzesicki *et al.*, 1991; Morzycki & Issekutz, 1991). Fibroblasts, and fibroblast-like cells are constituents of the connective tissue matrix and interactions between these and

immune cells are therefore likely to be of influence in both the infiltration and perpetuation of cells at an inflammatory site.

The pattern of integrin expression in bone (Clover *et al.*, 1992) and cartilage (Salter *et al.*, 1992) has been defined. The receptor αVβ3 has been reported to play a central role in the resorptive process which is stimulated by retinoic acid and inhibited by oestrogen. In osteoclast precursors, oestrogen is able to inhibit $1,25(OH)_2D_3$-stimulated αV expression at the mRNA level (Medhora *et al.*, 1991). In contrast to its effects on αV, oestrogen enhanced $1,25(OH)_2D_3$-induced β3 mRNA in the same cell type (Chiba *et al.*, 1992). Oestrogen also increased β-subunit expression in endothelial cells, and could potentiate the effect of TNF on the endothelial cell monolayers in terms of inducing leukocyte adhesion and upregulating ICAM-1 and ELAM-1 (Cid *et al.*, 1992). Thus, there is a potential role for systemic hormones in regulating cell-cell and cell-matrix interactions via cell adhesion molecules, possibly by modulating the actions of other agents rather than a direct effect.

1.8 CYTOKINES AS POTENTIAL MEDIATORS OF SEX HORMONE EFFECTS

Cytokines represent a diverse network of communicative signals between cells of the immune system and between the immune system and other organs. These factors were originally designated monokines, ie. those originating from monocytes, and lymphokines, ie. those originating from lymphocytes. However, the term cytokine has now been adopted to encompass a vast array of protein mediators of diverse cellular origin and which have the potential to carry out a wide range of functions.

In an immune response, accessory cells release factors such as IL-1 which can function as T cell activators. This leads to T cell production of cytokines such as IL-2, IFN-γ and GM-CSF, which act on a range of cells to produce an 'inflammatory response'. These cells, in turn, secrete other cytokines including TNF-α, IL-4, IL-6 and interleukin-8 (IL-8). At the same time the response can be downregulated, for example by IFN-γ and transforming growth factor-β (TGF-β), and the latter cytokine can also initiate reparative processes. This is, however, an oversimplified picture of a complex cascade of events which require equally intricate control mechanisms to prevent the response from continuing *ad infinitum* (reviewed by Panayi, 1990).

One possible method of dampening down an inflammatory response would be through the release of cytokine inhibitors or antagonists (reviewed by Arend & Dayer, 1990; Panayi, 1990). IL-1 inhibitory activity has been demonstrated in the urine from normal or febrile patients as well as in body fluids and cell supernatants. The factors reported have been of varying size and generally ill-defined. One such inhibitory activity however has been characterised. The 22 kD protein, originally defined as a product of human monocytes cultured on adherent human IgG, was found to be a specific receptor antagonist and was hence named interleukin-1 receptor antagonist protein (IRAP) (Arend *et al.*, 1989). The antagonist was subsequently shown to be produced by cell lines of monocytic origin, mononuclear cells and lymphocytes following adherence to IgG or stimulation with PMA, GM-CSF, TGF-β1 or interleukin-3 (IL-3). IRAP gene expression and protein production has been detected in RA and OA synovium (Firestein *et al.*, 1992). In addition, soluble receptors for TNF, IL-6 and IL-2 have been described which are able to bind their respective ligands and hence reduce the levels of free, active cytokine. Conversely, autoantibodies to various cytokines have been reported, in the normal situation as well as in disease states (reviewed by Bendtzen *et al.*, 1990). It is thought that these may act as physiological carriers and regulators, with the production of specific autoantibodies heightened under pathological conditions to 'mop up' excess cytokines.

RA is one example of an uncontrolled inflammatory response which is accompanied by abnormal cytokine expression (reviewed by Arend & Dayer, 1990; Brennan *et al.*, 1991; Wilder *et al.*, 1991). Synovial cells produce virtually all cytokines known to date. However, the macrophage-derived products, such as IL-1, IL-6, TNF-α and IL-8, are present in abundance, whereas the T cell-derived cytokines, including IFN-γ, IL-2 and TNF-β, are detectable only at the mRNA level. Indeed, data from *in situ* hybridisation studies have suggested that the macrophage-like and fibroblast-like cells of the synovium are the primary sources of cytokines, rather than synovial T lymphocytes (Firestein *et al.*, 1990).

TNF-α, IL-1α, IL-1β, IL-6 and TGF-β have been detected in sections of inflamed synovial tissue from RA patients, mainly localised to the thickened lining layer of the synovial membrane, but all are also found in interstitial tissue and occasionally in association with endothelial cells (reviewed by Brennan *et al.*, 1991). Where there was a distinct cartilage/pannus junction (CPJ), observed in areas of active joint destruction, TNF-α, IL-1α, IL-6, GM-CSF and TGF-β1 were detected, whereas only TGF-β was found at the diffuse fibroblastic CPJ where reparative processes occur (Chu *et al.*, 1992). The former pattern of cytokines was also seen for chondrocytes from RA and

normal joints, suggesting that they may be involved in normal cartilage homeostasis, with proinflammatory cytokines such as IL-1 balanced by others such as TGF-β.

1.8.1 Interleukin-1

Interleukin-1 (IL-1) was originally termed 'endogenous pyrogen' due to its role as a heat-labile protein found in acute leukocytic exudate fluid which, when injected into humans or animals, induced fever. Other effects of IL-1 noted included hepatic acute-phase protein synthesis, neutrophilia and increased T cell responsiveness to antigen or mitogen *in vitro*. It is now known to elicit a wide range of biological effects (reviewed by Dinarello, 1989). The IL-1 family includes the antagonist IRAP and two proteins, IL-1α and IL-1β, which have distinct primary amino acid sequences but are structurally related. Mature IL-1 occurs as a 15-17 kD peptide with IL-1β being the secreted form and IL-1α remaining associated with the plasma membrane. Both forms are biologically active and bind to the same receptors, of which there are two classes, types I and II, of high and low affinity, respectively. The receptors are members of an immunoglobulin superfamily (reviewed by Foxwell *et al.*, 1992). Type I receptors are found on T cells, fibroblasts, monocytes, synovial lining cells, keratinocytes, endothelial cells and chondrocytes, whereas type II are expressed on B cells, macrophages and neutrophils. IL-1 is structurally similar to the acidic fibroblast growth factor (aFGF) and shares many biological responses with TNF and IL-6, the three cytokines being the mediators of systemic 'acute phase' responses. IL-1 and TNF in particular share an almost identical spectrum of effects, often act synergistically, and are involved in self-augmentation.

In the immune system IL-1 stimulates T cell activation leading to IL-2 production and the expression of IL-2 receptors and acts as a cofactor during B cell activation, especially in concert with IL-4. It can also activate NK cells and potentiate macrophage cytotoxicity, and is responsible for inducing the production of an array of other cytokines. In addition, IL-1 has haematologic, metabolic, vascular and neurological actions. Its potent catabolic effects on bone and cartilage, including the release of degradative enzymes such as collagenase, proteoglycanase and other metalloproteinases, and the activation of osteoclasts, suggests a direct role in bone and joint disorders such as RA. The ability to augment fibroblast proliferation and collagen synthesis and induce vascular changes also suggests IL-1 as a likely participant in pannus formation. Indeed, the intra-articular injection of IL-1 into rabbit knee joints was accompanied by a cellular infiltration and loss of proteoglycan (Pettipher *et al.*,

1986) and repeated injections resulted in a chronic synovitis within those joints previously subjected to an antigenic challenge, pannus formation and joint destruction (Stimpson *et al.*, 1988).

IL-1 activity has been reported in the synovial fluid from various arthritic states including RA (Nouri *et al.*, 1984). However, early observations were made using the classical thymocyte bioassays, later shown to be highly susceptible to IL-6 which may have accounted for the results obtained. Hopkins *et al.* (1988) used the D10 bioassay and radioimmunoassay to confirm that IL-1 activity was indeed present in RA synovial fluid, although the presence of IRAP still makes quantitative analysis difficult. Both IL-1α and IL-1β have been localised to the RA synovial membrane and CPJ, as previously described, and primary cultures of human rheumatoid synovial cells were found to express binding sites for the two isotypes (Chin *et al.*, 1988). In addition, Deleuran *et al.* (1992) demonstrated the presence of IL-1 type I receptor and IRAP in the synovial membrane. However, IRAP was virtually absent at the CPJ where there was abundant IL-1α and IL-1 type I receptor, suggesting that insufficient production of the antagonist may contribute to the cartilage destruction occurring. High levels of IL-1α and IL-1 type I receptor with correspondingly low levels of IRAP were also measured in endothelial cells, further evidence for the importance of IL-1 in cellular migration into the synovium (see Section 1.7). This group also showed that areas staining for IL-1α overlapped with those showing high levels of TNF-α, supporting an interactive role for these two cytokines (see Section 1.8.2).

In the quest for the mechanism behind the bone-sparing effects of oestrogen in OP, Pacifici *et al.* (1989) discovered that the IL-1 activity in the monocyte conditioned media (MCM) from untreated postmenopausal OP patients was significantly higher than in the MCM from either premenopausal females or treated postmenopausal OP patients. Whereas in the non-OP group IL-1 levels declined back to premenopausal levels within eight years of the menopause, the OP patients had continuing elevated IL-1 levels. Treatment with a combination of oestrogen and progesterone for one month was associated with a reduction in IL-1 activity in all postmenopausal females tested. Subsequently, it was demonstrated that PBMC cytokine production, including IL-1, IL-6, TNF-α and GM-CSF, increased in parallel with various indices of bone resorption following oophorectomy in a group of premenopausal females (Pacifici *et al.*, 1991a; Pioli *et al.*, 1992). Oestrogen replacement therapy reversed these abnormalities (Pacifici *et al.*, 1991a) and either oestrogen or an IL-1 receptor antagonist were effective in reducing bone loss in ovariectomised rats (Kimble *et al.*, 1992). More recently it has been shown that bone matrix fragments, released during increased bone

turnover, have the ability to stimulate IL-1 release from PBMC, suggested to be due to a direct contact between the bone-derived factors and PBMC via integrin receptors on the cell surface (Pacifici *et al.*, 1991b; Pacifici *et al.*, 1992a)

In addition to the above results demonstrating that ovarian steroids can reduce postmenopausal increases in IL-1, progesterone and oestrogen have also been shown to increase monocyte IL-1 activity (Polan *et al.*, 1988; Polan *et al.*, 1989). However, the stimulation occurred at low hormonal concentrations and at higher doses an inhibition was seen. In a study of non-OP postmenopausal women, Stock *et al.* (1989) found that oestrogen only reduced IL-1 levels that were abnormally high at the start of treatment, suggesting a selective hormonal action. Rather than a role for IL-1 in mediating bone loss, it is now thought that inadequate secretion of IL-1 receptor antagonist, IRAP, could be a causal factor, and oestrogen in turn may correct abnormal secretion of this controlling factor (Pacifici *et al.*, 1992b).

1.8.2 Tumour Necrosis Factor

Tumour necrosis factor (TNF), a peptide of 17 kD, was identified as being analogous to 'cachectin', a macrophage-derived hormone which could induce cachexia, a wasting syndrome in mice, following injection of endotoxin. It was later renamed for its ability to cause the haemorrhagic necrosis of tumours and is now known to elicit an array of biological effects (reviewed by Beutler & Cerami, 1987; Vassalli, 1992). Many of its effects, such as osteoclast activation, synovial cell prostaglandin E_2 (PGE_2) and collagenase production and the induction of other cytokines, are shared with IL-1 (reviewed by Dinarello, 1989). Recently, the use of anti-TNF-α antibodies has been shown to inhibit synovial cell IL-1 production and it has been suggested that, in RA, TNF-α may be the main inducer of IL-1 activity (Brennan *et al.*, 1989). However, TNF may have beneficial as well as deleterious effects in autoimmunity (reviewed by Jacob, 1992). For example, a deficiency in TNF production has been suggested to be one of the contributory factors in the B/W lupus model and also in human SLE, and treatment with TNF-α delayed the onset of nephritis. In addition, TNF-α can both synergise with IFN-γ and antagonise IFN-γ-mediated upregulation of MHC class II expression. There is also some evidence that this cytokine is involved in the development of self-tolerance.

The genes for TNF (TNF-α) and lymphotoxin (LT or TNF-β), a tumourolytic protein derived from lymphocytes, are closely HLA-linked on chromosome 6, the only known

cytokine genes to date located within the MHC. TNF-α and -β bind to the same receptor and appear to evoke similar biological responses. TNF-α is primarily produced by stimulated monocytes or macrophages, but can also derive from lymphocytes, PMNL, mast cells, endothelial cells and keratinocytes. It is synthesised as a prohormone and this synthesis pathway is tightly controlled. There are two TNF receptors of 55 and 75 kD, types I and II, with different intracellular regions which may represent alternative signalling pathways. The two receptors are part of a family of proteins of similar structure, including CD27 and the Fas antigen (reviewed by Foxwell *et al.*, 1992). Both receptors exist in soluble forms identified *in vivo*, in the plasma of normal individuals and in even higher amounts in inflammatory states, the formation of which seems to be by proteolytic cleavage of the mature cell surface receptor, similar to the generation of the soluble IL-2 receptor α-chain (see Section 1.8.5). It has been postulated that these may serve a dual role, to absorb out and inactivate excess TNF, and to act as a slow release reservoir (reviewed by Chouaib *et al.*, 1991).

TNF-α, but not TNF-β, has been found in the synovial fluid and sera of RA patients and the levels of this cytokine appeared to correlate with disease activity (Saxne *et al.*, 1988). In one study of sex hormone effects on immune cell TNF production, Ralston *et al.* (1990) found that 17β-oestradiol could inhibit TNF production from unstimulated peripheral blood mononuclear cells (PBMC), but in postmenopausal women only. In contrast, hydrocortisone was effective in all groups tested, whereas DHT was inactive. The majority of postmenopausal women included in this study were suffering from OP, hence the effects of oestrogen in this group could have been as a result of the presence of OP, or due to the underlying hormonal deficiency.

1.8.3 Interleukin-6

Interleukin-6 (IL-6) was originally termed IFN-β2 or B cell stimulatory factor-2. It is now known to function in the more general regulation of immune and acute phase responses and haematopoiesis, with effects which include the induction of IL-2 production and IL-2 receptor expression by T cells, and enhancement of IL-3-induced multi-potential colony cell formation (reviewed by Hirano, 1990; Hirano *et al.*, 1990; Van Snick, 1990). IL-6 is produced by a wide variety of cell types, including T and B cells, monocytes, fibroblasts, endothelial cells and bone marrow stromal cells. The IL-6 receptor, a glycoprotein of 80 kD, is a member of the haematopoietic growth factor receptor family, and is found on resting T cells, but only on activated B cells. Other receptors of the same family include the β– and γ-chains of the human IL-2

receptor, that for human GM-CSF and murine IL-4, IL-3 and erythropoietin receptors (reviewed by Foxwell *et al.*, 1992). It also has several features in common with the GH and prolactin receptors. Further studies on the IL-6 receptor revealed an auxillary signalling molecule, gp130 (β-chain). This molecule can associate with the protein/receptor complex to convert the receptor from a low to high affinity state. The soluble IL-6 receptor, produced naturally, can also bind IL-6 and mediate an effect.

Increased levels of IL-6 have been reported in both the serum and synovial fluid of patients with RA and OA, and *in situ* hybridisation of IL-6 mRNA demonstrated positive cells in both lymphocyte-rich aggregates and adjacent to small blood vessels in the synovium (Wood *et al.*, 1992). The predominant source appeared to be the synovial T cell, and the close proximity of these cells with CD14+ tissue macrophages suggested that cell-cell contact may be required to initiate the production of IL-6. Both TNF and IL-1 are known to be potent inducers of IL-6 and could provide the signal from macrophage to T cell. IL-1 potentiates IL-6 mRNA expression and protein secretion in both human bone (Linkhart *et al.*, 1991) and cartilage (Nietfeld *et al.*, 1990). This could provide a means of amplifying the effects of IL-1, as IL-6 is required for IL-1-induced inhibition of proteoglycan synthesis, for example (Linkhart *et al.*, 1991), but IL-6 alone does not seem to induce human bone cell resorption (Nietfeld *et al.*, 1990; Littlewood *et al.*, 1991).

Rifas *et al.* (1992) were able to inhibit IL-1- and TNF-induced IL-6 secretion from human osteoblast-like cells with physiological concentrations of 17β-oestradiol, an effect which was repeated in the same cells, and also in bone marrow-derived stromal cells, with testosterone and progesterone as well as 17β-oestradiol (Girasole *et al.*, 1992). Moreover, ovariectomy in mice was found to be associated with increases in colony forming units-granulocyte/macrophage, thought to be the osteoclast progenitor cell, as well as giving rise to neutrophils and monocytes, an effect which could be reversed by treatment with either 17β-oestradiol or a mAb against murine IL-6 (Jilka *et al.*, 1992). The mechanism for this regulation of IL-6 by oestrogen was discovered to be via an ER-mediated effect on the transcriptional activity of the IL-6 promoter in stimulated cells only (Pottratz *et al.*, 1992). Therefore, it seems that in an oestrogen-deficient state the upregulation of IL-6 expression and production by immune cells and bone-derived cells could lead to osteoclastogenesis and the amplification of other cytokine responses, culminating in bone resorption.

1.8.4 Transforming Growth Factor

Transforming growth factors (TGF)-α and -β are distinct polypeptides with unique biological activities and separate receptors. TGF-α binds to the epidermal growth factor (EGF) receptor, and both the sequence and biological effects of TGF-α are homologous to those of EGF. TGF-β represents a large family of factors with diverse actions on a wide range of target tissues and cells (reviewed by Massague, 1990). There are now known to be at least five isotypes, TGF-β1-β5, and a heterodimer between TGF-β1 and -β2 (TGF-β1.2) also exists. TGF-β is a 25 kD homodimer which was initially described by its ability to induce the phenotypic transformation of non-neoplastic cells in culture. It is released as part of an inactive complex which requires prior activation and release of the TGF-β molecule before receptor interactions can occur. The distribution of this cytokine is widespread with the richest cellular source being the platelet. It is also found in large quantities in bone. In contrast, TGF-α has a very limited location and is not found within the cytoskeleton. The initial observations were of a growth differentiating, morphogenetic role for TGF-β. However, it has since become clear that it can act in opposing ways to either inhibit or stimulate proliferation and is actually one of the most potent growth inhibitors known. Additionally, it has important roles in embryogenesis, fibrosis and angiogenesis.

TGF-β has been identified as a crucial factor in the control of inflammation, immune regulation and tissue repair (reviewed by Wahl *et al.*, 1989; Palladino *et al.*, 1990), with the monocyte/macrophage playing a central role in these proceedings (reviewed by Wahl *et al.*, 1990). Theoretically, platelets arriving at an inflammatory site release TGF-β which results in the infiltration of other cell types, such as the monocyte, via its chemotactic properties. The general production of inflammatory mediators would cause monocyte activation, for example TGF-β is able to upregulate FcγRIII (CD16) expression (Welch *et al.*, 1990), leading to increased phagocytic and lysosomal activity and the release of reactive oxygen intermediates. TGF-β is equally able to suppress an inflammatory response, and can subsequently initiate tissue repair by stimulating the release of other growth factors, and controlling cell adhesion by increasing the production of extracellular matrix components and inhibiting their degradation, and by stimulating the expression of various cell adhesion receptors. It acts to cause immunosuppression, for example by inhibiting IL-2-dependent T cell proliferation and IL-2 receptor expression (Kehrl *et al.*, 1986), and by depressing NK cell activity (Ortaldo *et al.*, 1991). Pretreatment with TGF-β generally suppresses stimulated cytokine production *in vitro* (Chantry *et al.*, 1989; Brennan *et al.*, 1990; Turner *et al.*, 1990), although it has also been reported to have a potentiating effect at the

transcriptional level (Chantry *et al.*, 1989), and can enhance the translation of certain factors, such as IL-6 (Turner *et al.*, 1990), which may mediate some of its effects. Moreover, TGF-β can autoregulate its own production, which occurs in activated monocytes (Wahl *et al.*, 1990) and which is likely to be important in sustaining levels of this cytokine in an inflammatory response.

The presence of TGF-β in the synovial fluid and its production by synovium is well documented (Brennan *et al.*, 1990; Brennan *et al.*, 1991). *In situ* hybridisation techniques have localised the production in RA synovial tissues to fibroblast-like synoviocytes in particular, indicating that this cytokine plays a pivotal role in the development of this disease (reviewed by Wilder *et al.*, 1990). Indeed, the induction of FcγRIII expression on synovial mononuclear phagocytes from RA patients appears to be due, at least in part, to TGF-β (Wahl *et al.*, 1992), which may contribute to the tissue damage occurring as a result of the release of toxic oxygen metabolites. It has also been suggested that TGF-β may result in the impaired mitogenic responses and phenotypic changes seen, such as the inversion of the CD4:CD8 ratio via a selective inhibition of CD4+ cell proliferation (Lotz *et al.*, 1990), and may hence account for most of the immunosuppressive activity within the synovial fluid. However, as synovial cells are pre-activated the effectiveness of TGF-β may be reduced (Brennan *et al.*, 1990). Nevertheless, the administration of TGF-β1 protected against CIA (Kuruvilla *et al.*, 1991) and could be crucial to repair within the RA joint, both via its effects on matrix synthesis and as a potential 'coupling factor' between bone resorption and formation (reviewed by Centrella *et al.*, 1988).

The effects of the steroid hormones on TGF-β physiology are more widely reported than for any other cytokine (reviewed by Wakefield *et al.*, 1990). In breast cancer cells oestrogens and/or progestins can modulate the production of TGF-α (Liu *et al.*, 1987) and TGF-β (Jeng & Jordan, 1991), which in turn regulates tumour growth and differentiation. *In vivo* oestrogen is reported to increase TGF-β production by osteosarcoma cells (Komm *et al.*, 1988) and TGF-β itself appears to stimulate bone formation (Noda & Camilliere, 1989). However, effects *in vitro* are somewhat confusing and are dependant on parameters such as the cell type studied and other growth factors present. Oestrogen-induced release of TGF-β in human osteoblast-like cells was found to be less in the presence of PTH, itself a potent inducer of bone resorption and TGF-β production (Keeting *et al.*, 1989; Oursler *et al.*, 1991). Thus, the ability of oestrogen to reduce bone resorption may involve the release of TGF-β from bone cells. In support of this theory, Finkelman *et al.* (1992) reported that ovariectomy reduced the concentration of TGF-β in rat long bones and this could be reversed by

treatment with 17β-oestradiol. When studying human osteoblast-like osteosarcoma cells it was found that the androgens DHT and testosterone also increase TGF-β expression (Benz *et al.*, 1991), and may therefore, act like oestrogens to inhibit bone resorption.

1.8.5 Interleukin-2

Interleukin-2 (IL-2) was originally discovered for its mitogenic effects on T cells and hence received the name 'T cell growth factor' (Morgan *et al.*, 1976). Upon activation, T cells were found to secrete IL-2 and express IL-2 receptors, which led to an autoinduction loop and resulted, along with cytokines such as IL-4 and IL-6, in the clonal expansion of B cells and antibody formation (Robb *et al.*, 1981).
IL-2 exists as a 15 kD peptide and the receptor is composed of at least three distinct chains, α, β and γ (reviewed by Minami *et al.*, 1993; Taniguchi & Minami, 1993). The α-chain is a 55 kD membrane glycoprotein (p55), also known as the Tac protein or CD25. The gene encoding the α-chain is undetectable in resting T cells and is induced upon activation. The gene for the 70 kD β-chain (p70) is expressed constitutively in CD8+ but not CD4+ T cells, and that for the 64 kD γ-chain (p64) is expressed constitutively in all lymphoid cells. The γ-chain can combine with the β-chain alone to form a receptor with intermediate binding affinity, or with both the α- and β-subunits to form a high-affinity complex, whereas the α-chain alone forms a receptor of low affinity. The β-chain is critical for IL-2-mediated signal transduction and causes rapid tyrosine phosphorylation, and recent evidence suggests that the γ-chain is essential for ligand internalisation.

Interestingly, monocytes have now been found to express the p75 IL-2 β-receptor and respond to exogenous IL-2 with a general increase in activity, including the induction of IL-1 and TNF mRNA, peroxide production and an enhanced microbicidal and tumoricidal potential (Espinoza-Delgado *et al.*, 1990). IL-2 was also found to increase monocyte IL-6 production which was independent of the IL-1-induced effect, suggesting two separate pathways for expanding the immune response. Only the latter pathway was blocked by IFN-γ, whereas both could be blocked by the addition of TGF-β, which may represent a 'safety valve' mechanism against continued stimulation (Musso *et al.*, 1992).

The production of IL-2 in response to PHA has been shown to be low in T lymphocytes from the peripheral blood, synovial fluid or synovial cells in RA (Combe *et al.*, 1985). RA cells were also poor responders in *in vitro* assays of cell-mediated

immune responses which could be reversed by the addition of IL-2 (Emery *et al.*, 1984). The enzyme polyamine oxidase (PAO) metabolises the polyamines spermine and spermidine into hydrogen peroxide, ammonia and different aldehydes. It is possible that the products of this reaction inhibit IL-2 production. In support of this theory, Flescher *et al.* (1989) found that polyamine levels were elevated in RA patients and a negative correlation between IL-2 production and the concentration of polyamines in RA synovial fluid MNC was found (Flescher *et al.*, 1992). In addition, the direct interaction between PAO and its substrates was able to inhibit T cell IL-2 production (Flescher *et al.*, 1991), and by blocking the production of polyamines with an ornithine decarboxylase (ODC) inhibitor, or by blocking polyamine oxidation with an inhibitor of PAO, IL-2 production by the rheumatoid cells was seen to increase (Flescher *et al.*, 1992). Catalase was also effective, providing further evidence for a detrimental effect of the oxidation products. As oestrogen has been shown to stimulate the release of prolactin, which would subsequently increase the production of polyamines, it is possible that depressed levels of IL-2 may be mediated by the sex hormones (see Section 1.8.6).

An alternative explanation for the reduced IL-2 production, and/or diminished responsiveness to IL-2, is the existence of an inhibitor. Miossec *et al.* (1987) reported the presence of an inhibitor of IL-2 in the synovial fluid from RA patients. This was later identified as the soluble form of the IL-2 receptor, high levels of which have been measured in both the serum and synovial fluid from RA patients (Miossec *et al.*, 1990). The soluble receptors are formed from the proteolytic cleavage of the α-chain and, although these low affinity receptors appear unable to bind IL-2 to an extent which would interfere with its activity, they may represent a postactivation marker for T cells which are poorly responsive to IL-2 or be due simply to an increased T cell turnover.

1.8.6 Prolactin

Prolactin and growth hormone (GH) have highly homologous amino acid sequences and receptor structures and were originally thought to be one and the same hormone, indeed it is thought that the two hormones, plus placental lactogen, evolved from the same ancestral gene. It was eventually determined that the two hormones existed as separate entities, and the cDNA sequence for prolactin is now known (Cooke *et al.*, 1981). Hypothalamic dopamine negatively controls prolactin secretion from the pituitary. Prolactin induces a dose-dependent increase in ODC, a key growth-regulatory enzyme which is expressed rapidly when cells are recruited into the G_1 phase of the cell

cycle. ODC is the rate-limiting step in the production of polyamines which are involved with cell growth and division (reviewed by Russell, 1989).

The earliest observation of a link between pituitary hormones and the immune system came in 1930, when Smith reported that hypophysectomised animals demonstrated atrophy of their thymus glands. Hypophysectomised animals have since been shown to have deficient humoral and cell-mediated immunity which can be restored by administering either prolactin, GH or placental lactogen (Nagy *et al.*, 1983a). Dwarf animals, which lack prolactin as well as growth hormone (GH), are also compromised in their immune response, and altered cellular patterns, such as an increase in T-suppressor cells and a reduction in NK cells, has been reported (Gupta *et al.*, 1983). The effects of prolactin on the immune system are now widely cited (reviewed by Chikanza & Panayi, 1991; Gala, 1991; Jara *et al.*, 1991; Berczi, 1993). In addition, it has been shown that the gene for prolactin is found on the short arm of chromosome 6, which also carries the MHC (McMichael & McDevitt, 1977), thus suggesting an association between the control of prolactin biology and the control of immune cell function.

Bromocriptine, a dopamine agonist, was found to inhibit such immune reactions as antibody formation and *in vitro* lymphocyte reactivity in the MLR and, *in vivo*, the development of adjuvant arthritis and graft-versus-host reactions were also suppressed (Nagy *et al.*, 1983b; Hiestand *et al.*, 1986). This evidence, along with the finding that methylacetylenic putrescine, an inhibitor of ODC, prevented the development of CIA (Wolos *et al.*, 1990), suggests that prolactin-induced ODC activity could be an important event in the progression from immune stimulus to chronic inflammation. Indeed, ODC has been demonstrated as an integral event regulating lymphocyte differentiation, proliferation and function (Klimpel *et al.*, 1979).

Increased polyamine production due to excessive ODC activity has also been linked to ER dysfunction. Thomas *et al.* (1991) detected significantly higher DNA binding of uterine ER in BALB/c mice compared to the lupus-prone model MRL-lpr. This was reversed with the ODC inhibitor difluoromethylornithine (DFMO), indicating that the ER dysfunction could be due to an overactivity of ODC. In support of this theory, two- to six-fold higher polyamine levels were found in the MRL-lpr mice (Thomas *et al.*, 1991). It has previously been shown that naturally occurring polyamines play an important role in altering the physicochemical properties and enhancing DNA binding of the PR (Thomas & Kiang, 1988). Polyamines have also been shown to affect the structure and stability of oestrogen-ER complexes (Thomas & Kiang, 1987), and

elevated levels have been recorded in RA (Flescher *et al.*, 1989) and SLE (Puri *et al.*, 1978) patients. One theory is that the overproduction of polyamines arises due to oestrogen regulation of ODC activity. ODC has previously been shown to be upregulated by factors including hormones (Thomas *et al.*, 1989), thus initiating a feedback inhibition of ER.

In Section 1.4.1 it was mentioned that prolactin levels were found to be elevated in a group of male SLE patients (Lavalle *et al.*, 1987). This has been found to be the case in further studies, with one such investigation reporting hyperprolactinaemia in 22% of all SLE patients included and those without increased prolactin were in a clinical and immunological remission (Jara *et al.*, 1991). Thus, prolactin would seem to be an important factor in SLE, but such an association has not been shown in RA patients (Folomeev *et al.*, 1990). However, Nagy *et al.* (1991) showed that whereas immunoreactive prolactin was normal in RA patients, there was a significant deficiency in the bioactivity of circulating prolactin which was more evident in males than females. The increased polyamine levels reported in SLE (Puri *et al.*, 1978) and RA (Flescher *et al.*, 1989) may be thought of as being due to prolactin-induced increases in ODC activity.

Despite the conflicting evidence, as prolactin is thought to have immuno-potentiating actions, a group of patients with active RA were treated with bromocriptine in an attempt to suppress the inflammatory response. However, the drug appeared to have only weak second-line effects (Marguerie *et al.*, 1990). Prolactin levels have been reported to be greater during pregnancy in SLE than in RA patients or healthy controls, with levels correlating with disease activity (Jara-Quezada *et al.*, 1991). It is possible therefore, that prolactin is one of the contributory factors in pregnancy-induced exacerbation of SLE and one could hypothesise that the postpartum flare in RA may also be attributed, at least in part, to surges in prolactin. In support of this theory, McMurray *et al.* (1991) measured a hyperprolactinaemia in B/W mice postpartum, and Mattson *et al.* (1991) stated that two hormonal changes were assumed critical for the postpartum flare of CIA in DBA/1 mice, the sudden drop in steroid hormone levels from those attained during pregnancy, and the surges of prolactin at, and after, delivery. Bromocriptine given to arthritic mice postpartum inhibited the clinical exacerbation of CIA (Whyte & Williams, 1988).

Human peripheral blood T cells, B cells and monocytes and spleen cells have been demonstrated to possess prolactin receptors coupled to ODC (Russell *et al.*, 1984a; Russell *et al.*, 1985). Subsequently, a 46 kD prolactin-like protein was found to be

synthesised by ConA-activated murine splenocytes (Montgomery *et al.*, 1987). This is considerably larger than anterior pituitary-derived prolactin of 24 kD. mRNA from rat lymphocytes hybridised to a rat prolactin cDNA, and this demonstrated a larger transcript than pituitary prolactin mRNA, indicating that the protein originating from cells of the immune system is a separate entity. Antibodies to pituitary prolactin have been shown to inhibit the response of various immune cells to a range of stimuli (Hartmann *et al.*, 1989) and to block the response of a murine T-helper cell line to IL-2 (Clevenger *et al.*, 1990). Prolactin itself is able to induce the expression of IL-2 receptors on the surface of lymphocytes (Mukherjee *et al.*, 1990), and it has been suggested that IL-2 itself can increase prolactin receptors and cause the release of sequestered prolactin which, when bound to its receptor, becomes internalised and translocates to the nucleus where it elicits cellular proliferation (Clevenger *et al.*, 1990). Thus it was hypothesised that prolactin may be important in IL-2-mediated cell cycle progression.

These results suggest that the ability of T lymphocytes to respond to an antigenic challenge may depend on the presence of pituitary-derived prolactin bound to cell-surface receptors and that, when stimulated, the cells produce a prolactin-like substance which can amplify the response of the same or other immune cells. Hence, prolactin has been termed a new cytokine. It has been postulated that the immunosuppressive activity of cyclosporine is due, at least in part, to its ability to block prolactin actions on lymphoid organs (Russell *et al.*, 1984a; Russell *et al.*, 1985) and to inhibit T lymphocyte proliferation by reducing the intracellular action of prolactin (Russell *et al.*, 1987). Cyclosporine has been demonstrated to inhibit ODC induction in response to prolactin in rat tissues and it was therefore proposed as a prolactin antagonist (Russell *et al.*, 1984b). Hiestand *et al.* (1986) found that prolactin could compete with cyclosporine for a common binding site on rat T lymphocytes and that stimulation of prolactin secretion reversed cyclosporine-induced immunosuppression.

There is increasing evidence to suggest that the intracellular action of prolactin involves the activation of PKC (Russell *et al.*, 1987; Russell *et al.*, 1989). As previously described, incubation of PBMC with the enzyme PAO and its polyamine substrate, spermine, resulted in decreased IL-2 production (Flescher *et al.*, 1991) (see Section 1.8.5). This was accompanied by reductions in both PKC activity and the phosphorylation of tyrosine residues on endogenous proteins. The mobilisation of internal calcium was also suppressed. Results suggested that RA T lymphocytes showed an inherent reduction of internal calcium signalling, which may reflect a baseline increase in polyamines, due in turn to over-production of prolactin. In the Nb2

rat T lymphoma cell line prolactin is mitogenic and induces the transcription of early T cell activation genes, such as c-*fos*, c-*myc* and hsp70 (Stevens & Yu-Lee, 1991). Alternatively, rather than a direct effect on T cells, prolactin may act indirectly by altering the balance of circulating T cell subsets as there have been reports of an increase in the number of CD5+ and CD8+ cells following prolactin administration (De Bernardi *et al.*, 1990; Mukherjee *et al.*, 1990).

The data discussed support a direct role for prolactin in the pathogenesis of autoimmune conditions such a SLE and RA. However, the defect may not occur at the level of this hormone but may be due to an underlying defect in oestrogen metabolism. Oestrogen has been shown to induce prolactin release, probably via a direct effect on the pituitary (Franks, 1983). An increase in prolactin mRNA expression has been detected in anterior pituitary lobes from male rats treated with oestrogen and this was blocked by using an anti-calmodulin agent suggesting that the calcium-calmodulin system may be involved (Martinez-Campos *et al.*, 1991).

1.8.7 Other Cytokines

Interferon-γ (IFN-γ) is produced by certain classes of lymphocyte in response to an antigenic challenge. IFNs have been widely used as chemotherapeutic agents due to their ability to inhibit the growth of neoplastic as well as normal cells. However, apart from its growth-inhibitory function, its role appears to be mainly stimulatory. Indeed, IFN-γ is an important controlling factor in immunity with effects such as the promotion of lymphocyte responses, macrophage activation, induction of MHC expression and the production of other cytokines (reviewed by Trinchieri & Perussia, 1985).

The observation that lymphocytes from female mice secrete higher amounts of IFN-γ following immune stimulation than the cells from male mice, both *in vivo* and *in vitro* (Huygen & Palfliet, 1984), implied that this cytokine may be under hormonal control. This is supported by the work of Fox *et al.* (1991) who concluded, from transfection studies, that 17β-oestradiol could upregulate the activity of the IFN-γ promoter via regions in the 5'-flanking region of the IFN-γ gene which resembled the ERE. DHT has been shown to inhibit anti-CD3-induced IFN-γ production from murine T cells *in vitro*, whereas DHT given in combination with dehydroepiandrosterone (DHEA) was able to restore T cell IFN-γ producing capacity in older mice to that of the younger population (Araneo *et al.*, 1991). Therefore, there would seem to be, in animal models at least, a complex interaction between various sex steroid metabolites and cells of the

immune system in controlling the production of cytokines such as IFN-γ, and hence in influencing immune potential.

In addition to the above mentioned cytokines, granulocyte-macrophage colony-stimulating factor (GM-CSF) activity has been detected in rheumatoid synovial effusions and in the supernatants from cultures of RA synovial tissues (Xu *et al.*, 1989), although the effect of sex hormones on GM-CSF production and effects have yet to be investigated. Thus, there are a vast array of cytokines which have important enhancing or suppressive roles in an inflammatory response, and sex hormones have the potential to modulate the release or the subsequent effects of these factors.

To date the literature is abundant in clinical and epidemiological studies showing an association between heightened immune function in females and increased susceptibility to autoimmunity, with trends observed between hormonal fluctuations and alterations in immune function or disease activity. However, research which helps to explain the actual mechanism of action of sex hormones is inconclusive and often contradictory. This project was formulated to investigate the cellular effects of sex hormones with specific reference to their possible role in RA. The importance of cytokines is now well established and this array of proteins, in association with the family of adhesion molecules, are likely to play a key role in the pathogenesis of the disease. Thus, the research strategy was directed towards the effects of sex hormones, in particular oestrogen, on cytokine production and adhesion molecule expression by human and rheumatoid PBMC. In addition, receptors for oestrogen in these cells were studied in an attempt to clarify a somewhat confounding literature.

2.1 MATERIALS

All standard reagents were obtained from Sigma (Poole,UK) or BDH (Lutterworth, UK). Details of the origin of the more specialised reagents used are listed below, grouped into categories which appear alphabetically.

2.1.1 Antibodies

All antibodies used were either ascites purified or supplied as a culture supernatant.

Monoclonal antibodies (mAb) UCHM1 and UCHT1 raised against CD14 and CD3, respectively, were kind gifts from Prof. P. Beverley, ICRF (London,UK). Anti-CD19 was a kind gift from Prof. I. MacKlennan, University of Birmingham Medical School (Birmingham,UK).

MAbs to oestrogen receptor were purchased from Biogenesis Ltd. (Bournemouth,UK) and mAb ER-D5, raised against an oestrogen receptor-related antigen, was obtained from Amersham International plc. (Amersham,UK). Anti-topoisomerase and anti-centromere antibodies were prepared by Dr. J. Whyte from the sera of systemic sclerosis patients.

T52/7 antibody, directed against the α1 (CDw49a) subunit of integrin α1β1, was a generous gift of Dr. Hemler (Boston,MA) and mAb 23C6, directed against αV subunit (CD51), was a kind gift from Dr. Horton (London,UK). MAbs raised against α2 (CDw49b), α4 (CDw49d), α5 (CDw49e), αL (CD11a) and β2 (CD18) were purchased from Immunotech (Birmingham,UK). MAbs against α3 (CDw49c), α6 (CDw49f), αM (CD11b) and β1 (CD29) were supplied by Bioquote Ltd. (Ilkley,UK), Serotech Ltd. (Oxford,UK), Dakopatts (High Wycombe,UK) and Coulter Immunology (Luton,UK), respectively.

2.1.2 Cytokines

Recombinant human interleukin-6 (rhIL-6) was a generous gift from Dr. L. A. Aarden of the Netherlands Red Cross Blood Transfusion Service. A specific activity of 10^6 U/μg was determined using the B9 hybridoma cell bioassay. Recombinant human tumour necrosis factor α (rhTNFα) was a generous gift from Dr. G. R. Adolf of

Boehringer Ingelheim. The specific activity was determined using a murine L-M cell bioassay at $6X10^7$ U/mg. Recombinant human interleukin-2 (rhIL-2) was donated by Glaxo with a pre-determined specific activity of 10^6-10^7 U/mg.

2.1.3 cDNA Probes

IL-6 cDNA was a kind gift from J. Brakenhoff, Netherlands Red Cross Blood Transfusion Service. IL-1β and TGF-β cDNA probes were generous gifts from Dr. A. Shaw and Dr. A. Baxter, both of Glaxo Group Research. The cDNA probe for TNF-α was a kind gift from Dr. S. Ralston, Rheumatic Diseases Unit, Northern General Hospital (Edinburgh,UK). A prolactin cDNA probe was generously provided by Dr. D. Savva, Department of Biochemistry and Physiology, University of Reading (Reading,UK) and a cDNA probe for oestrogen receptor was a kind gift from Dr. G.L. Greene, Ben May Laboratory for Cancer Research, University of Chicago (Chicago,USA). GAPDH cDNA was a generous gift from Dr. J. Beresford, now at Bath Institute for Rheumatic Diseases, and the probe for β-actin was present in-house.

A summary of cDNA probes used and details such as the vector in which they are contained is shown in Section 2.8.7.

2.1.4 Hormones

17α- and 17β-oestradiol, testosterone and tamoxifen (citrate salt) were obtained from Sigma. Follicle-stimulating hormone (FSH) was purchased from Calbiochem Novabiochem (UK) Ltd. (Nottingham,UK).

2.1.5 Immunochemicals

All FITC-conjugated antibodies and immunoglobulins were purchased from Sigma. R-Phycoerythrin-conjugated monoclonal mouse anti-human B-cell (CD19), T-cell (CD3) and monocyte (CD14) antibodies were obtained from Dakopatts (High Wycombe,UK). Human AB serum and normal rabbit serum were obtained from GIBCO (Paisley,UK). FITC coated latex beads were from Flow Cytometry Standards (Research Triangle Park, NC,USA) and sheep anti-mouse IgG Dynabeads from Dynal (UK) Ltd.

(Wirral,UK). Reagents for APAAP staining were purchased from Dakopatts and Diff-Quik reagents were from Baxter Dade AG (Dudington, Germany).

2.1.6 Kits

The Abbott ER-ICA Monoclonal for detecting oestrogen receptors was supplied by Abbott Laboratories Ltd. (Maidenhead,UK), as were control slides for the kit. DNA was purified using either the Magic Minipreps DNA Purification System from Promega Ltd. (Southampton,UK), which relies on a reverse-phase method of extraction, or the Qiagen Plasmid Kit distributed by Hybaid Ltd. (Teddington,UK) which uses an ion-exchange method to purify DNA. DNA was then further purified using either a Geneclean II or Mermaid Kit, distributed by Stratech Scientific Ltd. (Luton,UK). cDNA was labelled with [α-^{32}P]dCTP using a Multiprime Kit from Amersham International plc. (Amersham,UK). RNA was further purified to mRNA using the Promega PolyATtract mRNA Isolation System by Promega Ltd. An AmpliTaq Cycle Sequencing Kit was purchased from Perkin-Elmer Ltd. (Beaconsfield,UK).

2.1.7 Molecular Biology Reagents

All restriction enzymes were purchased from either Northumbria Biologicals (NBL) (Cramlington,UK) or Pharmacia Biosystems Ltd. (Milton Keynes,UK). M-MLV and SuperScript RNase H$^-$ reverse transcriptases were supplied by GIBCO (Paisley,UK) and Taq DNA polymerase by either NBL, Promega Ltd. (Southampton,UK) or Perkin-Elmer Ltd. (Beaconsfield,UK). pd(T)$_{12-18}$, RNAguard RNase inhibitor and an Ultrapure dNTP set were obtained from Pharmacia Biosystems Ltd. DNA markers were supplied by either NBL or Promega Ltd. Bacto-agar,-yeast extract and -tryptone were obtained from Oxoid (Bristol,UK). Standard agarose was purchased from Sigma and NuSieve 3:1 agarose from FMC Bioproducts, the UK distributor of which is Flowgen (Sittingbourne,UK). Water-saturated phenol (AquaPhenol) was supplied by Appligene (Co. Durham,UK). All oligonucleotide primers were synthesised, according to instructions, by Colin Lazarus, Department of Botany, University of Bristol (Bristol,UK).

Hybond-N+ was supplied by Amersham International plc. (Amersham,UK) and DE81 discs and chromatography paper (3MM) were from Whatman Scientific Ltd. (Maidstone,UK).

2.1.8 Radiochemicals

[2,4,6,7-^{3}H]oestradiol and [^{3}H]thymidine were from Amersham International plc. (Amersham,UK). [α-^{32}P]dCTP was obtained from ICN Flow (High Wycombe,UK) and [γ-^{32}P]dATP from NEN-Dupont (UK) Ltd. (Stevenage,UK). Scintillation fluid utilised included Optiscint HiSafe and Optiphase HiSafe 3 from Wallac Ltd. (Milton Keynes, UK).

2.1.9 Tissue Culture Reagents

RPMI 1640, Minimal Essential medium (MEM), penicillin-streptomycin, L-glutamine, sodium bicarbonate and trypsin-EDTA were all obtained from GIBCO (Paisley,UK). Heat-inactivated foetal calf serum (FCS) was obtained from Imperial Laboratories (Andover,UK) and for the preparation of charcoal-stripped FCS, high-grade charcoal (Norit GSX) from BDH was utilised. Phosphate buffered saline tablets and Lymphoprep were purchased from Oxoid (Bristol,UK) and Nycomed (UK) Ltd. (Birmingham,UK), respectively. Heparin (Monoparin) was supplied by CP Pharmaceuticals Ltd. (Wrexham,UK)

ZR-75 cells were a kind gift from Dr. T. Ruprinak, Glaxo (Greenford,UK) and Hs578T cells were purchased from the European Collection of Animal Cell Cultures (ECACC) (Salisbury,UK). Synovial fibroblasts were generously donated by Dr. N. Jordan, University of Bath (Bath,UK). All other cell types included in this study were used routinely in our laboratory.

2.2. PREPARATION OF MEDIA AND BUFFERS

RPMI 1640 / Minimal Essential medium (MEM) + 10% (v/v) foetal calf serum (FCS)

RPMI 1640 or MEM (10X)	50 ml
Penicillin (10000 IU/ml) / Streptomycin (10000 μg/ml)	5 ml
Glutamine (200 mM)	5 ml
Sodium bicarbonate (7.5% (w/v))	15 ml
Heat-inactivated FCS	50 ml

The above reagents were combined and made up to 500 ml with sterile micronised water. The pH was adjusted to 7.3 with sterile 3 M NaOH.

Phenol red-free RPMI 1640 / MEM + 10% (v/v) charcoal-stripped FCS

This was made up as described above, using RPMI or MEM devoid of phenol red (10X), which is reported to be weakly oestrogenic (Berthois *et al.*, 1986), and using FCS which had been charcoal-stripped to remove endogenous steroids (see below).

Serum-free RPMI 1640 / MEM

RPMI 1640 or MEM (10X)	50 ml
Penicillin (10000 IU/ml) / Streptomycin (10000 μg/ml)	5 ml
Glutamine (200 mM)	5 ml
Sodium bicarbonate (7.5% (w/v))	15 ml
Transferrin	500 μl
Insulin (5 mg/ml)	100 μl
Sodium selenite ($3X10^{-4}$ M)	50 μl
BSA	0.1% (w/v)

Transferrin was first preloaded with $FeCl_3$ by mixing 100 mg transferrin with 100 μl $FeCl_3$ (10^{-2} M in 1 mM HCl) and 9.9 ml of MEM then leaving for 30 minutes at room temperature. The solution was filter sterilised and stored at 4°C. Insulin was dissolved in 10 mM acetic acid to 5 mg/ml and stored at -70°C. Sodium selenite was dissolved in PBS at a concentration of $3X10^{-4}$ M. To make up the media, all reagents were mixed

and the volume made up to 500 ml with sterile micronised water. The pH was adjusted to 7.3 with sterile 3 M NaOH.

Charcoal-Stripped FCS (CS-FCS)

To 100 ml heat-inactivated FCS was added 4 g of high grade charcoal (Norit GSX) and the mixture was stirred, on ice, for 1 hour. Charcoal was separated from the serum by centrifuging at 3000 rpm for 10 minutes, and the supernatant was then filtered through 0.4 μm pre-filters followed by 0.2 μm filters. The serum was re-sterilised by passing through a sterile 0.2 μm filter and stored at -20°C.

Phosphate Buffered Saline (PBS)

5 PBS tablets were dissolved in 500 ml micronised water and the solution autoclaved. For PBS-G, 0.8 mg medicinal glucose was dissolved in a small volume of sterile PBS then filtered back into the buffer.

Tris Buffered Saline (TBS)

Sodium chloride	8 g (0.14 M)
Potassium chloride	0.2 g (0.0026 M)
Tris base	3 g (0.025 M)

The above reagents were dissolved in 800 ml DH_20, 0.015 g phenol red added and the pH adjusted to 7.4 with HCl. Finally, the volume was made up to 1 litre with DH_20.

Tris-Borate-EDTA (TBE) (5X)

Tris base	54 g (0.445 M)
Boric acid	27.5 g (0.445 M)
EDTA (0.5 M, pH 8.0)	20 ml (0.01 M)

The above reagents were dissolved in DH_20 and made up to a final volume of 1 litre.

Tris-Acetate-EDTA (TAE) (10X)

Tris base	48.44 g (0.4 M)
Sodium acetate	4.1 g (0.05 M)
EDTA (0.5 M, pH 8.0)	3.72 g (0.01 M)

The above reagents were dissolved in DH_20 to a final volume of 1 litre and the pH corrected to 8.1 with glacial acetic acid (approximately 12 ml required for 1 litre of 10X solution).

MOPS (10X)

MOPS	41.85 g (free acid) or 46.2 g (Na salt) (0.2 M)
Sodium acetate	4.1 g (anhydrous) or 6.8 g (trihydrate) (0.05 M)
EDTA	3.72 g (0.01 M)

The above were dissolved in 1 litre of DH_2O and the pH adjusted to 7.0 with NaOH. The solution was then autoclaved.

Transformation Buffer 1 (Tfb1)

Potassium acetate	0.03 M
Rubidium chloride	0.1 M
Calcium chloride	0.01 M
Manganese chloride	0.05 M

15% (v/v) glycerol was added and the pH adjusted to 5.8 with 0.2 M acetic acid. The solution was then filter sterilised.

Transformation Buffer 2 (Tfb2)

MOPS	10 mM
Calcium chloride	75 mM
Rubidium chloride	10 mM

15% (v/v) glycerol was added and the pH adjusted to 6.5 with KOH. The solution was then filter sterilised.

2.3 PREPARATION OF DRUG SOLUTIONS

17α-oestradiol, 17β-oestradiol, testosterone and tamoxifen were dissolved in ethanol to a concentration of 1 mM, as required. Further dilutions were made in the relevant culture medium.

Follicle-stimulating hormone (FSH) was dissolved in PBS (pH 7.6) to a concentration of 200 μg/ml. Aliquots were stored at -70°C for up to 6 months.

2.4 TISSUE CULTURE

2.4.1 Preparation of Peripheral Blood Mononuclear Cells

Peripheral blood was obtained from normal, healthy volunteers or from rheumatoid arthritis patients attending The Royal National Hospital for Rheumatic Diseases. Blood was routinely collected between 9.00 a.m. and 12.00 p.m. A record was kept for female donors regarding menopausal status, stage of menstrual cycle, parity and use of oral contraceptives. Patients used for this study had no history of steroidal use. Most were receiving non-steroidal anti-inflammatory drugs (NSAIDs) and a small number were also treated with a second-line agent.

Blood obtained was dispensed into heparinised tubes (100 U/ml). After diluting 1:1 with PBS-G, the blood was separated by density-gradient centrifugation on Lymphoprep (density = 1.077 g/ml). The mononuclear cell (MNC) layer was extracted from the serum/lymphoprep interface and cells were washed twice in PBS-G, then counted using white cell counting fluid (3% (v/v) glacial acetic acid, 0.1% (w/v) methylene blue).

2.4.2 Purification of T Cells and Monocytes

PBMC prepared as described in Method 2.4.1 were divided into two 15 ml polypropylene tubes and pelleted by centrifuging at 1000 rpm for 10 minutes. Cells

were resuspended in either 500 µl anti-CD14 and 5 µl anti-CD19 mAbs, to purify the T cell population, or 500 µl anti-CD3 and 5 µl anti-CD19 mAbs, to purify the monocyte population. The cells were incubated at 4°C , with rotation, for 1 hour, then washed twice in medium (RPMI supplemented with 10% (v/v) FCS) and resuspended in sheep anti-mouse IgG-coated Dynabeads (diluted 1/10 in medium). After a further 1 hour incubation at 4°C, with rotation, the cell populations of interest were negatively selected, by magnetically drawing the Dynabeads to the sides of the tubes and carefully removing the supernatants. The two cell populations were washed twice in medium and counted prior to use.

2.4.3 Maintenance of Cell Lines

B9 Cells

B9 is a murine myeloma cell line. The cells were maintained in RPMI supplemented with 5% (v/v) FCS. To 30 ml of cells were added 15 µl β-mercaptoethanol (β-ME) and 30 µl monocyte conditioned media (MCM) (prepared by incubating MNC in 10 ml media supplemented with 10% (v/v) FCS for 24 hours, centrifuging at 1000 rpm for 10 minutes, and removing the supernatants which were stored at -20°C).

Fibroblasts

Synovial fibroblasts were grown from synovial explants taken from osteoarthritis patients. They were maintained in MEM with 10% (v/v) FCS up to a passage number of approximately 10.

Hs578T Cells

Hs578T is a human breast carcinoma cell line of epithelial origin which is oestrogen receptor negative. The cells were maintained in RPMI supplemented with 10% (v/v) FCS and 10 µg/ml bovine insulin.

T-47D Cells

The T-47D cell line was established from the pleural effusion of a ductal carcinoma of the breast of a 54 year old female, and is oestrogen receptor positive. The cells are maintained in RPMI with 10% (v/v) FCS.

WEHI 164 Cells

WEHI 164 subclone 13 is a mouse fibrosarcoma cell line. The cells are maintained in RPMI with 10% (v/v) FCS.

ZR-75 Cells

The ZR-75 cell line was established from a malignant ascitic effusion in a 63 year old female Caucasian with infiltrating ductal carcinoma. Cells are of epithelial origin and are oestrogen receptor positive. They were maintained in RPMI with 10% (v/v) FCS, supplemented with 10^{-9} M oestradiol.

2.4.4 Passaging Cells

Adherent cells were passaged as follows. The medium was removed and the cell monolayer rinsed with PBS. 2.5 ml trypsin-EDTA was added to each flask and the cells then incubated at 37°C for 5-10 minutes. To ensure that all cells were dislodged, the flask was tapped gently, then 10 ml medium was added, the suspension removed and the cells pelleted by centrifugation. After washing twice in medium the cells were resuspended in medium and split (normally 1:4 to 1:10), then recultured to confluence.

Non-adherent cells were passaged by removing an aliquot of cell suspension from each flask and dispensing into flasks containing a relevant volume of culture medium.

2.4.5 Freezing Down Cells

Cells were harvested, washed and counted, then resuspended in 50% (v/v) FCS/50% (v/v) medium (either RPMI 1640 or MEM) to a concentration of approximately $10X10^6$ cells/ml. 500 µl aliquots of the cells were dispensed into freezing vials and placed on ice. 500 µl of 20% (v/v) dimethyl sulphoxide (DMSO)/80% (v/v) medium was added drop-wise to each vial, whilst mixing thoroughly. Vials were then placed into a polystyrene container packed with tissue. This was sealed and placed into a -70°C freezer overnight before transferring to liquid nitrogen storage.

To set up cells from frozen, a vial was removed from liquid nitrogen storage and defrosted rapidly under hot running water. The outside of the vial was then wiped with alcohol prior to removing the cells. Cells were washed twice with medium then grown

up overnight in a small (25 cm^2) tissue culture flask before transferring to a larger (75 cm^2) flask.

2.4.6 Assessing Cell Viability

Cell viability was routinely assessed using the trypan blue exclusion method, by which the cell suspension was mixed with an equal volume of trypan blue and incubated for between 5 and 15 minutes, at 37°C. Cells were then counted and those which had taken up the blue stain (ie. those which were non-viable) were expressed as a fraction of the total number of cells present (ie. viable and non-viable), hence percentage viability could be calculated. In all experiments recorded, cell viability was found to be greater than 90%.

2.5 BIOASSAYS

2.5.1 IL-6 Bioassay (Helle *et al.*, 1988)

The IL-6 bioassay utilises the B9 murine hybridoma cell line. B9 cells are a variant of the B13.29 line and proliferate in response to IL-6. Sensitivity for IL-6 is virtually 100%, except for a slight stimulatory effect with IL-4.

For the bioassay, 10 ml of murine B9 cell suspension was removed and the cells washed twice in RPMI 1640 supplemented with 5% (v/v) FCS, then resuspended to a concentration of $5X10^4$ cells/ml in medium supplemented with 50 μM β-ME. Supernatant samples were diluted in a 48-well plate to give final dilutions of 1/100, 1/1000, 1/10000, 1/100000 and 1/1000000. Cells were seeded into flat-bottomed 96-well plates at a concentration of $5X10^3$ cells/well. To the cells were added 100 μl aliquots of the sample dilutions, each performed in duplicate. A standard curve was performed with rhIL-6, diluted to give final concentrations of 100, 30, 10, 1 and 0.1 U/ml. Plates were incubated for 72 hours at 37°C, 5%CO_2/95%air. 20 μl 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml in PBS) was then added to each well, and the incubation continued for a further 2 hours. MTT is a pale yellow substrate which produces a dark blue formazan product when incubated with living cells. Once a formazan residue had formed, the supernatant was aspirated and 100 μl acidified isopropanol (99 ml propan-2-ol + 1 ml HCl) added to each well to dissolve the product. Absorbances were read on a Dynatech Automated Micro ELISA

reader at 570 and 630 nm, reference and test wavelengths, respectively, against blanks consisting of acidified isopropanol only.

The dilution giving a reading on the linear portion of the standard curve was selected and an extrapolation made to give U/ml IL-6, hence total IL-6 per sample could be calculated by multiplying by the relevant dilution factor. A typical standard curve for the IL-6 bioassay is shown in Figure 2.1.

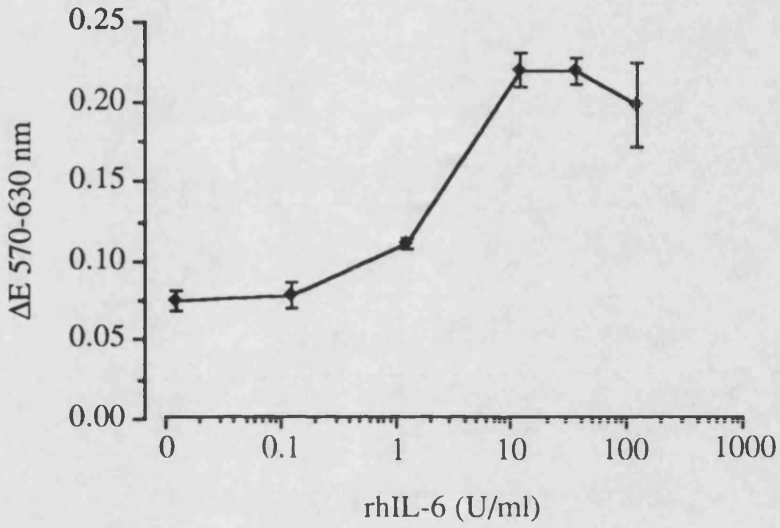


Figure 2.1. A typical standard curve for the IL-6 bioassay.

2.5.2 TNF Bioassay (Espevik and Nissen-Meyer, 1986)

The TNF bioassay utilises WEHI 164 subclone 13 cells, derived from WEHI 164 mouse fibrosarcoma cells. Both TNF-α and TNF-β are cytotoxic for this particular cell line.

For the bioassay, WEHI 164 cells were prepared to give a concentration of $2X10^5$ cells/ml in RPMI 1640 supplemented with 10% (v/v) FCS. Cells were seeded into 96-well flat-bottomed plates and incubated for 3-4 hours at 37°C, 5%CO_2/95%air, until the cells had adhered. 1/100 dilutions of supernatant samples were added to the cells, with each dilution performed in triplicate. A standard curve was performed with

rhTNFα at 0.001-1000 U/ml. Plates were incubated for 24 hours at 37°C, 5%CO_2/95%air. 10 µl MTT (5 mg/ml in PBS) was then added to each well and the incubation continued for a further 4 hours. Finally, the supernatant was aspirated and 50 µl acidified isopropanol added to each well to dissolve the formazan residue. Absorbances were read at 570 and 630 nm, reference and test wavelengths, respectively, against blanks consisting of acidified isopropanol only.

Results were expressed as % cytotoxicity using the formula:

$$\frac{(1 - \text{O.D. standard/sample})}{(\text{O.D. zero standard})} \times 100$$

Extrapolations were made from the linear portion of the standard curve and multiplied by the dilution factor to give total U/ml TNF per sample. A typical standard curve for the TNF bioassay is shown in Figure 2.2.

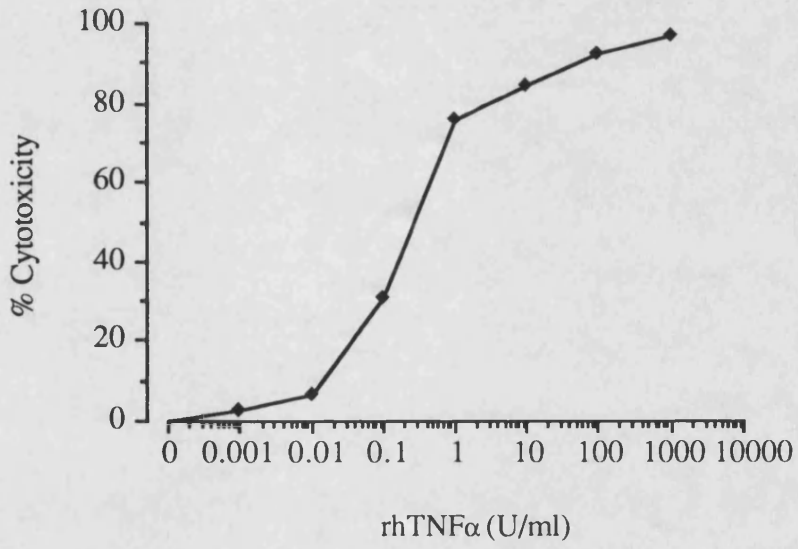


Figure 2.2. A typical standard curve for the TNF bioassay

2.6 PROLIFERATION ASSAY

Cells were harvested, washed twice in relevant medium and resuspended to a concentration of $1X10^6$ cells/ml. The cells were then aliquotted into the wells of a 96-well round-bottomed plate ($1X10^5$ cells/well) along with the stimuli and/or inhibitors of proliferation under study. Cells were incubated at 37°C, 5%CO_2/95% air, for 72 hours, pulsed with [^{3}H]thymidine (1 μCi/well) and incubated for a further 4 hours. Cells were harvested onto glass filters using a Skatron Semiautomatic cell harvester. The filters were then dried and immersed in 500 μl of Optiscint HiSafe scintillation cocktail, in scintillation vials. Counts were made in a LKB 1209 Rackbeta liquid scintillation counter.

2.7 IMMUNOCYTOCHEMISTRY

2.7.1 Preparation of Cells for the Analysis of Cell-Surface Antigens by Flow Cytometry.

Cells were harvested, washed twice in medium, counted and resuspended to a concentration of $1X10^6$ cells/ml. 100 μl aliquots of cell suspension were dispensed into FACS tubes and 2 ml PBS/FCS (2% (v/v) FCS in PBS) added. Cells were pelleted by centrifuging at 1000 rpm for 5 minutes and the supernatant aspirated using a suction pump. The cell pellet was then resuspended in 50 μl mAb (diluted appropriately in PBS/FCS) and 10 μl of human AB serum, with the latter added to block Fc receptors on the cell surface. Controls were performed by adding either PBS/FCS in place of the mAb, or the relevant immunoglobulin (IgG), diluted to the same concentration as the highest of mAb. Cells were incubated for 60-90 minutes on ice, then washed once in 3 ml PBS/FCS and harvested by centrifuging at 1000 rpm for 5 minutes, at 4°C. 50 μl of the relevant anti IgG FITC-conjugate (1/20 in PBS/FCS) was added and the cells incubated for a further 30 minutes, on ice. The cells were washed as previously described and resuspended in 200 μl PBS/FCS and 200 μl 2% (w/v) paraformaldehyde in PBS (pH 7.4), then stored at 4°C, in the dark, for up to 2 weeks prior to analysis.

For dual-labelling of cells an additional first antibody was included which was directly conjugated to a different fluorochrome, namely phycoerythrin.

2.7.2 Saponin-Permeabilisation of Cells for the Analysis of Intracellular Antigens by Flow Cytometry (Sander *et al.*, 1991)

Cells were prepared as described in 2.7.1 and resuspended to a concentration of $2X10^6$ cells/ml in PBS/BSA (0.1% (w/v) bovine serum albumin (BSA) in PBS). Cells were aliquotted into FACS tubes ($1X10^5$ cells/tube) and an equal volume of 2% (w/v) paraformaldehyde solution added in order to fix the cell membrane prior to it being rendered permeable. The cells were incubated for 5 minutes on ice, washed once in 500 µl PBS/BSA and harvested by centrifuging at 1000 rpm for 5 minutes, 4°C. The supernatant was removed by aspiration, 500 µl of PBS/saponin (0.1% (w/v) saponin in PBS/BSA) added to permeabilise the membrane, and the cells incubated on ice for a further 5 minutes. Following a second wash with PBS/saponin, 50 µl mAb (diluted appropriately in PBS/saponin) was added and the cells incubated for 60 minutes on ice. Controls were performed as in 2.7.1. After incubation with the first antibody, cells were washed once in PBS/saponin then 50 µl of the relevant anti-IgG FITC conjugate (diluted 1/100 in PBS/saponin) added, and the incubation continued for a further 30 minutes. Finally, the cells were washed once in PBS/saponin and then in PBS/BSA to 're-seal' the membrane. Cells were resuspended in 200 µl PBS/BSA and 200 µl 2% (w/v) paraformaldehyde and stored at 4°C, in the dark, for up to 2 weeks prior to analysis.

2.7.3 Analysis of Flow Cytometry Data

FACS samples were analysed on a Becton Dickenson FACStar Plus, equipped with a 100mW argon ion laser and Consort 32 computer. The principal of flow cytometry is that a single cell suspension passes through a light source emitted by a laser. The light excites the fluorescent dyes used to label the cells, leading to light amplification. Scattered or fluorescent light produced is collected by a photomultiplier tube (PMT) and converted to electrical signals and eventually to digital information. The light produced is reflected by mirrors, coated such that they reflect only a specific wavelength. Therefore, one cell preparation can be labelled with several different fluorochromes which all emit a different spectrum of light.

Prior to collecting data, the nozel head was aligned to maximise the detection of fluorescent events. This was carried out using a mixture of two latex beads coated with FITC of different intensities. When a quantitative analysis of data was required, in terms of 'sites per cell', a mixture of six beads were used, standardised in terms of six

different levels of fluorescence achieved by varying the number of FITC sites per bead. By plotting a histogram of fluorescence intensity and gating on each of the six peaks the mean fluorescence intensity (MFI) for each peak could be calculated. The MFI values were entered into a Spreadsheet computer program which extrapolated from a standard curve, as shown in Figure 2.3, to calculate antibody sites per cell from the MFI values obtained for particular FITC-labelled cells.

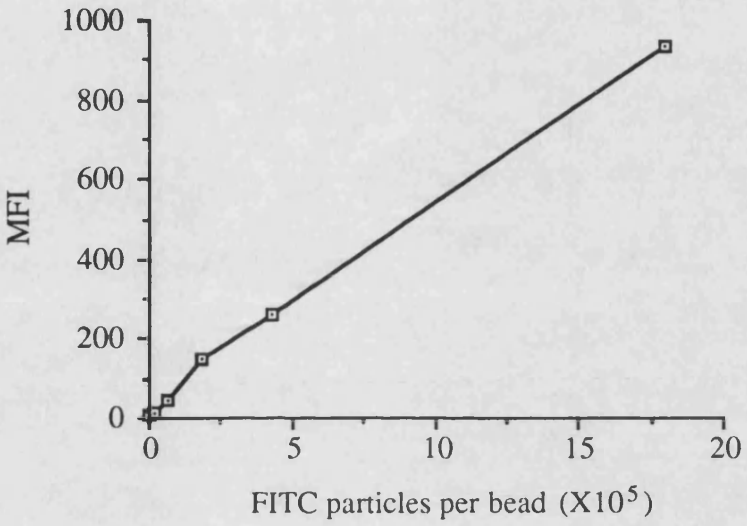


Figure 2.3. Standard curve for sites per cell analysis

Data were collected as either dot plots or frequency histograms. Dot plots usually consisted of side scatter (SSC), relating to cell granularity, versus forward scatter (FSC), relating to cell size, or of fluorescence versus FSC. Histograms generally depicted fluorescence intensity. The threshold was set such that background noise was eliminated. All parameters were first set using a negative control with second antibody (ie. FITC-conjugate) only. The remaining samples were then analysed, with all comparisons made with the relevant IgG control. Comparisons were made in terms of MFI values and % positive cells, with the control antibody set at 5%. Where quantitative analysis was performed the number of sites per cell for the IgG control were deducted from all other values.

2.7.4 Preparation of Slides

Use of the Abbott ER-ICA monoclonal kit for the detection of oestrogen receptors required that all slides be treated with the tissue adhesive included in the kit, according to instructions. For all other procedures either cytospins were performed with non-adherent cells, or for adherent cells, slides were treated with poly-L-lysine, to aid attachment, as follows. 100 µl/spot of 100 µg/ml poly-L-lysine was added to 4-spot slides, and the slides incubated for 5 minutes at 37°C. The slides were then washed three times in sterile micronised water before being left to dry in a sterile environment.

2.7.5 Preparation of Cells and Synovial Sections

Adherent cells were cultured onto slides by seeding at a density of approximately $2X10^4$ cells/spot of a 4-spot slide and incubating in a humidified chamber overnight at 37°C, 5%CO_2/95%air. Non-adherent cells were prepared as cytospins using a Shandon cytospin. Conditions were optimised as $2.5X10^5$ cells/slide, centrifuging at 500 rpm for 3 minutes. The Diff-Quik method of staining was used to show that cell morphology (nuclear and cytoplasmic) is maintained under these conditions (results not shown).

Synovial tissue was obtained from a 67 year old female rheumatoid patient with a 25 year history of RA. Tissue was removed from the active left knee which was described as warm and swollen on prior examination. The sample was snap frozen and stored in liquid nitrogen prior to cutting into 8 µm sections on a Bright's cryostat. The sections were placed onto slides which were either untreated, or which had been treated according to the Abbott kit instructions.

2.7.6 Staining for Oestrogen Receptor Using the Abbott ER-ICA Monoclonal Kit

Slides were fixed following instructions in the kit and stored at -20°C in the specimen storage medium described, for up to 4 months. The cells were then treated according to instructions, incubating with either the H222 anti-ER rat mAb or the control normal rat antibody. Abbott control slides were used to ensure that the procedure was working optimally. To produce a more intense staining, the incubation times were increased from 30 to 60 minutes.

Cells were examined using a Leitz Laborlux S microscope equipped with a Leitz Wild 46/52 Photoautomat.

2.7.7 Alkaline Phosphatase-Antialkaline Phosphatase (APAAP) Staining

4-spot slides onto which cells were cultured overnight were drained to remove excess medium, rinsed in TBS and fixed in acetone for 5 minutes. Slides were then wrapped in aluminium foil and stored at -20°C prior to use. Cytospins and cryostat sections were fixed directly in acetone for 1 minute or 10 minutes, respectively, and stored as described above.

The APAAP procedure was carried out according to Dakopatts' instructions. Briefly, slides were immersed in TBS for 5 minutes then blocked with normal rabbit serum (NRS) (20% (v/v) in TBS) for 20 minutes. The slides were 'flicked' to remove excess NRS and the primary mAb or a control IgG added, at an appropriate dilution in TBS. After 30 minutes incubation in a humidified chamber, at room temperature, slides were washed for 1-2 minutes in TBS prior to adding the second antibody (diluted 1/25 in TBS) and incubating for a further 30 minutes. After washing, the APAAP complex (1/50 dilution in TBS) was added for 30 minutes and, after washing once again, the alkaline phosphatase substrate (substrate 1, see below) was applied to the slides for 15-20 minutes. Finally, slides were washed in TBS, then in tap water, counterstained with haematoxylin for 30 seconds and mounted in 2% glycerol/PBS. Cells were examined as described in Method 2.7.6.

Substrate 1

Naphthol AS-MX phosphate	2 mg
Dimethylformamide	0.2 ml
0.1 M TRIS buffer pH 8.2	9.8 ml
1 M Levamisole	10 µl
Fast-Red TR salt	10 mg

The solution was prepared by dissolving the Napthol AS-MX phosphate in dimethylformamide in a glass tube and diluting to 10 ml with TRIS buffer. Levamisole was added to block endogenous alkaline phosphatase activity. Substrate 1 was stored at -20°C. The Fast-Red salt was added immediately prior to use and the solution filtered directly onto the slides.

2.8 ANALYSIS OF RNA AND DNA

The preparation of RNA requires that all solutions be sterile and treated with diethlypyrocarbonate (DEPC) to eliminate RNase activity.

2.8.1 RNA Extraction (Chomczynski & Sacchi, 1987)

Cells were lysed in solution D (see below) (10^6 cells/100 µl) and transferred to microfuge tubes. 50 µl 2 M sodium acetate pH 4.0, 500 µl water-saturated phenol (AquaPhenol) and 100 µl chloroform-isoamyl alcohol (49:1) were added sequentially, mixing by inversion between each addition. Tubes were shaken vigorously for 10 seconds then cooled on ice for 15 minutes. After centrifugation at high speed (13000 rpm) in a microfuge for 10 minutes, the top aqueous layer was removed and an equal volume of chloroform-isoamyl alcohol added. Tubes were shaken vigorously once again then centrifuged at 13000 rpm for 5 minutes. A second extraction with chloroform-isoamyl alcohol was performed if necessary and the final aqueous layer was precipitated with an equal volume of isopropanol at -20°C overnight. RNA was then spun out at 13000 rpm for 10 minutes, the supernatant removed and the pellet redissolved in 150 µl solution D. The RNA was precipitated by adding 150 µl isopropanol and storing at -20°C overnight. Finally, the RNA was pelleted by centrifugation at 13000 rpm for 10 minutes, the pellet washed twice in 70% (v/v) ethanol and once with absolute ethanol, then dried down under vacuum prior to being resuspended in 200 µl DEPC water.

Solution D

Guanidinium thiocyanate	250 g
0.75 M Sodium citrate, pH 7.0	17.6 ml
10% (w/v) N-Laurylsarcosine (sodium salt)	26.4 ml

The above were dissolved in 293 ml sterile DEPC-treated water at 65°C and stored at 4°C. For use, solution D was prepared by adding 72 µl 2-mercaptoethanol to 10 ml of the stock solution, and stored for up to two weeks.

2.8.2 Quantification of RNA and DNA

10 µl aliquots of DNA and RNA samples were diluted to 250 µl in water and the absorbances read at 260 and 280 nm in a LKB Ultrospec II spectrophotometer. The ratios of A_{260}/A_{280} were calculated. These should be 1.8 for DNA and 2.0 for RNA, with lower values indicating impurities or contamination by phenol or protein. The concentration of DNA or RNA was calculated by the formulae:

$$A_{260} \text{ X } 40 \text{ X dilution factor} = \mu g/ml \text{ RNA}$$

$$A_{260} \text{ X } 50 \text{ X dilution factor} = \mu g/ml \text{ DNA}$$

The quality of RNA prepared was assessed by electrophoresing through a 1.2% (w/v) agarose gel (see section 2.8.3). This should show two clear ribosomal bands.

2.8.3 Agarose Gel Electrophoresis

Horizontal submarine gel electrophoresis was used for the analysis of DNA and RNA. Unless otherwise stated, 1% (w/v) agarose in TAE was used for DNA and 1.2% (w/v) agarose in TAE for RNA analysis. The agarose was added to the buffer and heated until melted then allowed to cool to approximately 60°C. Ethidium bromide was added to give a final concentration of 0.5 µg/ml. Ethidium bromide contains a planar group which intercalates between staked bases of single- or double-stranded DNA causing the fluorescent intensity of the dye to increase, hence the DNA or RNA product can be visualised under UV light. A perspex mould with two solid sides was prepared by forming the remaining two sides with masking tape and placing a comb approximately 1 cm from one end, ensuring that it was not touching the bottom of the mould. The cooled agarose was poured into the mould and allowed to set at room temperature. Once set, the comb was carefully removed and the gel placed into the manifold with the wells towards the cathode. The gel was then covered in 1X TAE, the running buffer. DNA samples were loaded in DNA loading dye (15% (w/v) ficoll, 0.25% (w/v) xylene cyanol, 0.25% (w/v) bromophenol blue) and for RNA samples an RNA loading dye was used (20% (w/v) ficoll, 0.2% (w/v) bromophenol blue). RNA was denatured at 65°C for 10 minutes then quenched on ice prior to loading onto the gel. The samples were electrophoresed at 60 V, unless otherwise stated, until the leading dye was at least three-quarters of the way down the gel. The gel was visualised using a UV

transilluminator. Appropriate DNA markers were used to assist in the detection of DNA fragments of a specific size.

2.8.4 Dot-Blot Analysis of RNA

Samples were standardised to contain equal quantities of RNA/50 µl. The 50 µl aliquots of RNA were mixed with 50 µl formamide and denatured at 65°C for 10 minutes then quenched on ice. 8 µl of 3 M sodium acetate, pH 5.2, was added and the volume made up to 200 µl with solution A (see below). Two-fold serial dilutions were made with solution A in a 96-well plate, on ice. The dot-blot manifold was soaked in 0.1% (v/v) DEPC water for 30 minutes prior to assembling with the Hybond-N+, which had been pre-wetted with DEPC-water, then with 20X SSC. 100 µl samples were applied by suction at a rate of approximately 100 µl/20 minutes, then washed with 100 µl/well solution A. The RNA was fixed onto the filter by soaking in fresh 50 mM NaOH for 5 minutes. After rinsing in 2X SSC, the filter was sealed in Saran Wrap and stored at 4°C.

Solution A

Formamide (pure)	12 ml
3 M Sodium acetate, pH 5.2	2 ml
DEPC-water	10 ml

The above reagents were mixed and stored for up to 2 weeks at 4°C.

2.8.5 Northern Blot Analysis of RNA (Herrin and Schmidt, 1988)

A formaldehyde gel was constructed using 1% (w/v) agarose in 1X MOPS (diluted from 10X stock using 1 part formaldehyde:6 parts water), with 1X MOPS used as a running buffer. 10 µg samples of RNA were dried down under a vacuum, resuspended in 10 µl loading buffer (50% (v/v) formamide (pure), 16.7% (v/v) formaldehyde in 1X MOPS), then denatured at 65°C for 10 minutes and quenched on ice. 1 µl RNA loading dye was added to each sample which were then loaded onto the gel. Small gels were run at a constant current of 30 mAmps for approximately 2 hours, and large gels at 75 mAmps for approximately 5 hours.

When the gel had finished running it was removed from the tank, trimmed to remove the wells, and measured to enable a piece of Hybond-N+ to be cut to the same size. A corner was cut from the gel and from the corresponding piece of Hybond-N+ to enable the orientation to be assessed after blotting. The Northern blot was constructed as shown in figure 2.4. Care was taken not to trap any air bubbles between the layers, and any gel and wicks visible were covered with cling film to prevent short-circuiting and evaporation of the buffer. The blot was run overnight then carefully dismantled. To check for ribosomal bands the filter was first fixed in 50 mM NaOH (RNA-side down), then rinsed in 2X SSPE before placing in 0.02% (w/v) methylene blue (RNA-side up) (ribosomal bands should appear within 3 minutes). Finally the filter was washed in 1X SSPE until the background staining faded, then sealed in Saran Wrap and stored at 4°C.

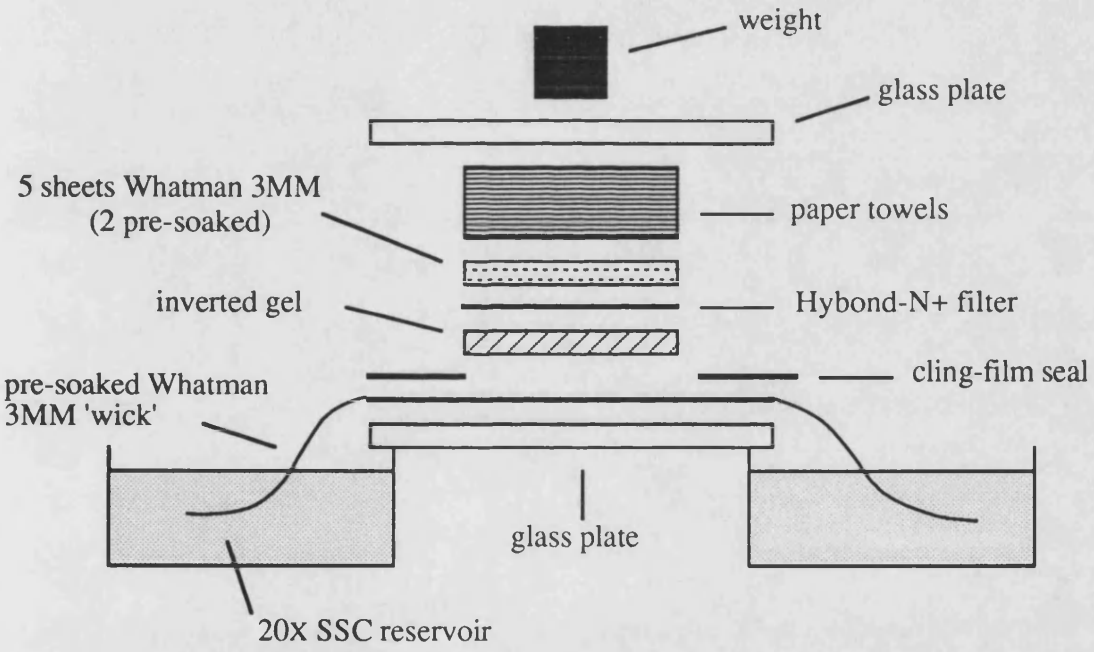


Figure 2.4. Northern Blotting

2.8.6 Southern Blot Analysis of DNA (Southern, 1975)

A restriction endonuclease digest was carried out initially, according to Method 2.8.7 (iv) to 'release' the fragment of DNA of interest. The DNA was electrophoresed on a 0.8% (w/v) agarose gel, with markers, then observed under UV light. The distance of the marker bands was measured for future reference. The gel was placed into 0.25 M HCl until the dyes had changed colour (bromophenol blue to green; xylene cyanol to yellow), rinsed in distilled water and left to soak in 0.4 M NaOH whilst the blotting apparatus was constructed (Figure 2.4). Blotting was carried out as for Northern blotting and the DNA allowed to transfer for 2-3 hours. The Hybond-N+ membrane was then removed, washed briefly by immersion into 2X SSPE, then wrapped in Saran Wrap and stored at 4°C.

2.8.7 Preparation of cDNA Probes

cDNA probes were generally supplied as recombinants. To maintain sufficient stocks of the cDNA, the plasmids were transformed into competent cells and stored at -70°C. Cells were then grown up from the frozen stocks, hence the amount of cDNA could be amplified. Table 2.1. summarises details of the probes used.

PROBE	NAME	VECTOR	RESISTANCE	ENZYME	SIZE
TNF-α	(PAT 153)	PAT 153	Tet.	PstI	1200
IL-1β	pGem1 IL-1β	pGem-1	Amp.	EcoRI+PstI	320
IL-6	(pSP64)	pSP64	Amp.	SalI+EcoRI	300
TGF-β	pGHTGFB-27	pGem	Amp.	HindIII+EcoRI	300
β-Actin	pHFbA-3'UT	pBR322	Amp.	EcoRI+BamHI	700
GAPDH	pG3PCR-4	pUC 18	Amp.	BamHI	1000
ER	pER35	pGem3	Amp.	SphI+EcoRI	1800
Prolactin	pJMBG61	pUR291	Amp.	PstI	588

Table 2.1. cDNA Probes

(i) Competent cells

A streak was made onto an agar plate from a frozen stock of bacteria, and incubated at 37°C overnight. A single fresh colony was then used to innoculate 5 ml of LB and the culture incubated at 37°C, with shaking, until the cells had reached early log phase of growth (O.D. 550 = 0.3). Bacteria were then subcultured (1:20) into 100 ml of LB, prewarmed to 37°C, and grown for a further 2 hours, or until the O.D. reading had reached 0.48. After chilling on ice, the culture was centrifuged in Corex tubes in a Beckman J2-21 centrifuge, at 5000 rpm for 10 minutes, 4°C. Cells were resuspended in 2/5 volume of Tfb1 and left on ice for 5 minutes. A second centrifugation was performed as before, then the cells were resuspended in 1/25 volume of Tfb2 and left on ice for a further 15 minutes. Finally, suitable aliquots were dispensed into pre-chilled freezing vials, snap frozen in dry ice and stored in liquid nitrogen.

(ii) Transformation of competent cells

Competent cells were placed at room temperature until just thawed, then left on ice for 10 minutes. Plasmid DNA was added up to 2/5 the volume of cells (no more than 100 ng/200 μl cells) and the cells incubated on ice for 30 minutes. The cells were heat shocked by placing at 42°C for 90 seconds then returned to ice for 2 minutes. Approximately 2 volumes of LB were added at room temperature and the cells then incubated at 37°C for 60 minutes with gentle shaking, to allow plasmid establishment. The culture was plated out onto agar plates, with the appropriate selection, and incubated for 30 minutes at 37°C, then the plates were inverted and the incubation continued overnight. A control culture was also performed whereby no DNA was added to the cells. This was plated out in the same manner (hence no colonies should form).

(iii) Plasmid preparation

Plasmid preparations were carried out on either a small or large scale, depending on the final quantity of DNA required. For small scale preparations, colonies from the above prepared agar plates (Section 2.8.7(ii)) were innoculated into 10 ml of LB containing either ampicillin or tetracycline (50 μg/ml) and incubated for 4-6 hours at 37°C, with shaking. A Magic Minipreps DNA Purification System was used according to the instructions in the kit, to purify plasmid DNA. For large scale plasmid preparations the

initial 10 ml culture was grown up as described above, then added to 490 ml of LB and the incubation continued overnight. The culture was spun at 4000 rpm for 10 minutes, 4°C, and the supernatant discarded. The plasmid DNA was then extracted from the bacterial pellet using a Qiagen Plasmid Kit, following the instructions for Maxi preparations. The concentration of DNA obtained was assessed as described in method 2.8.2.

(iv) Restriction endonuclease digestion

To 'release' the cDNA fragment from the plasmid in which it was contained a digest was performed. For this, purified DNA (volume equivalent to 1 μg) was mixed with the appropriate buffer (diluted 10-fold to its working concentration) and restriction endonuclease (1 U/μg DNA) in an eppendorf tube, ensuring that the enzyme did not exceed 10% of the final volume. After mixing thoroughly, the tube was placed at 37°C for 90 minutes. The reaction was stopped by placing the tube on ice and the sample analysed by agarose gel electrophoresis, as in method 2.8.3, with the undigested DNA included as a control.

When a double digest was carried out, both enzymes could be added together, provided that they shared a common buffer. If a separate buffer was required to ensure maximal efficiency of the second enzyme, then the sample buffer was adjusted after 60 minutes incubation with the first enzyme, and the incubation then continued at 37°C for a further 60 minutes with both enzymes.

(v) Purification of DNA from agarose

The fragment produced by restriction endonuclease digestion was excised from the agarose gel and purified using either a Geneclean Kit, designed to purify DNA of greater than 500 bp to 1.5 kb in length, or a Mermaid Kit , designed to purify DNA from 10 bp to 1 kb in length. Both kits utilise the fact that single and double stranded DNA is able to bind to the specially formulated silica matrix whilst DNA contaminants cannot. The resulting DNA was then analysed by agarose gel electrophoresis to ensure that it had been efficiently eluted from the silica matrix.

(vi) Verification of cDNA probes

cDNA probes require verification in terms of assuring that the probe is able to bind to the mRNA transcript of the correct size. This was carried out by labelling the cDNA as described in Section 2.8.8 and hybridising to a Northern blot filter, as in Section 2.8.9. The filter was then autoradiographed and the position of the resulting band(s) compared to the 18S and 28S ribosomal bands detected by staining the filter with 0.02% (w/v) methylene blue (Section 2.8.5).

2.8.8 Labelling of DNA

DNA was labelled using a Multiprime Kit based on the random sequence hexanucleotide priming method of Feinberg & Vogelstein (1983), using Klenow. Prior to labelling, the DNA sample (volume equivalent to 10-20 ng DNA) was diluted with sterile micronised water to give a final volume of 50 µl and heated at 95°C for 2-5 minutes to denature the DNA, which was then added to the standard Multiprime reaction and labelled for 3-5 hours, at room temperature, using [α-^{32}P]dCTP. The labelling reaction was terminated by the addition of 20 mM EDTA, and the volume made up to 100 µl with water.

To measure the incorporation of radioactive label into the DNA, 1 µl of the reaction mix was spotted onto the centre of each of two 2.3 cm discs of Whatman DE81 ion exchange paper. The paper is ionically charged such that DNA will bind but unincorporated nucleotides do not. One disc was washed three times, for 5 minutes each wash, in 0.5 M Na_2HPO_4, then rinsed in water and finally in absolute ethanol. Once dry, an approximation of the percentage incorporation could be made using a Series 900 mini monitor, but for an accurate assessment the filters were counted in a LKB 1209 Rackbeta liquid scintillation counter, using Optiphase HiSafe 3 scintillant.

2.8.9 Hybridisation

Prior to hybridisation, any residual methylene blue stain was removed from the surface of filters by washing in 0.2X SSPE, 1% (w/v) SDS for 15 minutes, with gentle agitation, according to the method of Herrin & Schmidt (1988). The prehybridisation solution was prepared as described below, and approximately 20 ml of the solution was used per hybridisation bottle. The filters were placed into the bottles with the

RNA/DNA side facing inwards, and incubated in a Hybaid rotary hybridisation oven, at 65°C overnight for Southern blots, and at 50°C for 3-5 hours for Northern blots and dot-blots. The labelled cDNA probe (Method 2.8.8) was denatured at 95°C for 5 minutes, quenched on ice, then added to the prehybridisation mix and the blots incubated at the above temperatures overnight.

Following the overnight incubation, the hybridisation fluid containing the probe was removed and stored at 4°C for future re-use. Southern blot filters were washed at 65°C, twice in 100 ml 2X SSPE, 0.5% (w/v) SDS for 15 minutes each wash, and once in 100 ml 0.2X SSPE, 0.5% (w/v) SDS for 10 minutes. Northern blot and dot-blot filters were washed twice in 100 ml 2X SSC, 0.5% (w/v) SDS, at 65°C, except when β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes were used, when higher stringency washes of 0.5X SSC, 0.1% (w/v) SDS were required. Blots were then wrapped in Saran Wrap and autoradiographed by exposing to X-ray film at -70°C, with an intensifying screen. Autoradiographs were analysed using a hand-held densitometer.

The radioactive probe was removed from the filters by placing them into a boiling solution of 0.1% (w/v) SDS and leaving until cool. Blots were then rinsed in 2X SSC, sealed in Saran Wrap and stored at 4°C.

Prehybridisation solution (Northern and dot blots)

50% (w/v) Dextran sulphate	10 ml (10%)
Formamide	16.5 ml (33%)
10X SSC	10 ml (2X)
20% (w/v) SDS	1.25 ml (0.5%)
0.5 M EDTA	200 µl (2 mM)
50X Denhardt's	10 ml (10X)
10 mg/ml Salmon sperm DNA	1 ml (0.2 mg/ml)

Denhardt's solution was prepared by dissolving 1% (w/v) ficoll, 1% (w/v) PVP and 1% (w/v) BSA in micronised water, filtering, and storing in aliquots at -20°C. A solution of 10 mg salmon sperm DNA/ml water was sheared by passing through a 19-gauge hypodermic needle several times. The solution was then denatured in a boiling water bath for 10 minutes, chilled quickly on ice, and stored at -20°C in small aliquots. The prehybridisation solution was then prepared by mixing all of the above reagents. The solution could be stored at room temperature for several months.

Prehybridisation solution (Southern blots)

30X SSPE	10 ml (6X)
50X Denhardt's solution	5 ml (5X)
50% (w/v) Dextran sulphate	5 ml (5%)
20% (w/v) SDS	2.5 ml (1%)
10 mg/ml Salmon sperm DNA	250 µl (50 µg/ml)
Water	27 ml

The solution was made up as described above.

2.8.10 Reverse Transcription of RNA

RNA was reverse transcribed to form cDNA prior to amplification in the polymerase chain reaction (PCR). Approximately 320 ng of total RNA, prepared as in Method 2.8.1, was mixed in an eppendorf tube with 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM each of dGTP, dATP, dTTP and dCTP, 1.5 µM $pd(T)_{12-18}$, 30 units RNAguard RNase inhibitor and 400 units M-MLV reverse transcriptase. The volume was made up to 30 µl with DEPC-water and the tube placed into a Perkin-Elmer Cetus DNA Thermal Cycler set at 37°C, for 60 minutes. When SuperScript RNase H- reverse transcriptase was used in place of M-MLV, the order of addition of reagents and temperatures used were followed according to instructions. The reaction was terminated by heating to 95°C for 10 minutes then placing on ice. Samples were either used directly in the PCR (Method 2.8.11), or were stored at -20°C for future use.

2.8.11 Amplification of DNA (Saiki *et al.*, 1988)

Amplification was carried out in a final volume of 100 µl containing 2 µl of the above reaction mix (Method 2.8.10), 200 µM each of dGTP, dATP, dTTP and dCTP, 1 µM of each of two oligonucleotides, specific for the DNA sequence, 2.5 units *Thermus aquaticus* (Taq) DNA polymerase (Chein *et al.*, 1976), $MgCl_2$ at its optimised concentration and 1X buffer, specific for the Taq DNA polymerase used. The mixture was overlayed with 100 µl of mineral oil to minimise evaporation, and amplification carried out in the Perkin-Elmer Cetus DNA Thermal Cycler. The DNA was denatured at 95°C, the oligonucleotides annealed at a temperature which depended on their

composition, and the DNA extended at 72°C. The length of each stage of the reaction and the number of cycles required to achieved maximal amplification depended on the primers used. Finally, 10 µl of the reaction mix was analysed by agarose gel electrophoresis, as in method 2.8.3. For a clearer resolution of bands, NuSieve 3:1 agarose was used, according to instructions.

2.8.12 Quantification of the PCR (Wiesner, 1992)

RNA was reverse transcribed as outlined (Method 2.8.10) and amplified by PCR using method 2.8.11 and including 100 µCi [α-^{32}P]dCTP in the PCR reaction. At alternate cycles, 2 µl of the reaction mix was removed and added to an eppendorf tube containing 5 µl of unlabelled PCR product, to facilitate visualisation of bands at early cycle number, and 3 µl water. Tubes were placed on ice to terminate the reaction and, once all samples had been collected, 1 µl DNA loading dye was added and the samples loaded onto a 4% (w/v) NuSieve 3:1 agarose gel. Bands were visualised under UV light and excised from the gel, then placed into scintillation vials. To measure background levels of radioactivity, slices were cut from nearer to the anode. The vials were left at 80°C overnight to dry the gel slices, 500 µl Optiphase HiSafe 3 added and counts made in a LKB 1209 Rackbeta liquid scintillation counter.

2.8.13 Sequencing

Sequencing reactions were performed using an AmpliTaq Cycle Sequencing Kit, which is based on the chain-termination DNA sequencing method (Sanger *et al.*, 1977). Primers were end-labelled with [γ-^{32}P]dATP using T4 polynucleotide kinase. Both the forward and reverse primers for the DNA under examination were labelled, and the forward primer alone from the kit was used with the control DNA. The cycling reaction was spiked with an additional 0.5 µl Taq DNA polymerase, and 1 µl [α-^{32}P]dCTP, to enhance the result.

Samples were analysed by denaturing gel electrophoresis, for which a denaturing gel was constructed as described below. Two glass plates, which had been soaked in 2 M KOH overnight, were rinsed in water and cleaned using absolute ethanol then air-dried. The top plate was treated with dimethyldichlorosilane solution to prevent the gel from sticking, then cleaned with water and ethanol as before. The bottom plate was treated on the gel-side with a solution of 20 ml ethanol, 120 µl glacial acetic acid and 200 µl

silane for 2 minutes, to facilitate the adherence of the gel, and was then cleaned once again with water and ethanol. The bottom plate was then prepared with spacers along its edges and the gel poured using the sliding plate technique. A shark's tooth comb was inserted into the gel upside-down and the plates clamped together until the gel had polymerised. Immediately prior to loading, the sequencing reactions were denatured at 75°C for 2 minutes. The comb was placed in the gel the right way up and 5 µl of reaction mix loaded into each well. The gel was run in TBE buffer at 1200 V, 30 mA for the appropriate amount of time. The top plate was then levered off and the gel soaked in 10% (v/v) methanol, 10% (v/v) HAc for 20 minutes. Finally, the gel was then drained and wrapped in Saran Wrap and autoradiographed overnight at room temperature.

Denaturing gel

Urea	21 g
40% (w/v) Acrylamide	10 ml
10X TBE	5 ml
16% (w/v) Ammonium persulphate	100 µl
N,N,N',N'-Tetramethylethylenediamine (TEMED)	100 µl

A 40% (w/v) solution of acrylamide was made by dissolving 38 g acrylamide (DNA-sequencing grade) and 2 g N,N'-methylenebisacrylamide in 100 ml water. This could be stored in the dark, at 4°C, for several weeks. The urea was then dissolved in the acrylamide solution and TBE by heating gently. The solution was cooled and, immediately prior to pouring the gel, the ammonium persulphate and TEMED were added.

2.9 STATISTICAL ANALYSIS

Data was expressed as mean ± SEM and analysed using an unpaired or paired Student's T-test where the number of experiments included did not exceed five. Where six or more experiments were included for analysis, the Mann-Whitney U test or Wilcoxon signed-rank test were employed. Each data point was compared separately to the control and corrections were not made for any statistical errors which may have thus arisen.

CHAPTER THREE

SEX HORMONE MODULATION OF CYTOKINE EXPRESSION

3.1 INTRODUCTION

The complex cellular interactions which occur during a normal inflammatory response appear to be governed, at least in part, by an equally complex array of inter- and intracellular mediators, including cytokines. The same factors are also likely to be involved in chronic inflammatory conditions such as RA. As was discussed in Chapter One, cytokine products such as IL-1α, IL-1β, TNF-α, IL-6 and TGF-β have been detected in the synovial fluid and synovial membrane from RA patients (reviewed by Brennan *et al.*, 1991), often in close proximity to areas of active joint destruction (Chu *et al.*, 1992). The release of such cytokines by cells within the joint may contribute to the pathogenesis of the disease. IL-1β and TNF-α, for example, share a vast spectrum of biological effects including the release of degradative enzymes, osteoclast activation and fibroblast proliferation (reviewed by Dinarello, 1989). Both IL-1 and TNF-α can induce IL-6 release which may act to amplify the destructive processes initiated by the latter two cytokines, as well as contributing with its own effects (reviewed by Van Snick, 1990). In addition, the cytokine TGF-β is critical to both the induction and potentiation of immune and inflammatory responses and also to the reparative processes which follow (reviewed by Wahl *et al.*, 1990).

The effect of sex hormones on cytokine production is not widely reported and the results that have appeared in the literature have been mainly concerned with hormonal modulation of bone cell cytokine expression and release. Therefore, experiments were designed to study the effects of the sex hormones oestrogen and testosterone on PBMC cytokine expression. PBMC were studied from pre-, peri- and postmenopausal female controls and RA patients in order to assess whether endogenous fluctuations in hormone levels also affect cytokine production. Cells were initially obtained from normal controls and cultured with various concentrations of 17β-oestradiol or testosterone. The release of IL-6 and TNF into the culture supernatant after 24 hours of incubation was studied by means of bioassays. Following this, a comparison was made between control and RA PBMC in terms of cytokine mRNA expression, using cDNA probes for IL-1β, TNF-α, IL-6 and TGF-β. Cells were cultured for differing lengths of time with a range of oestrogen concentrations. In addition, the expression of prolactin mRNA was studied in response to oestrogen treatment. Prolactin has been termed a new cytokine as it has the potential to act on human lymphocytes which express specific receptors (Russell *et al.*, 1984a), and these cells can then release a prolactin-like substance (Montgomery *et al.*, 1987) which also binds to the receptors on the surface of immune cells. It is thought to have an important role in immune function,

either *per se*, or through the induction of ODC and the subsequent production of polyamines (see Introduction, Section 1.8.5).

The release of oestrogen is under the control of FSH from the pituitary. As it is possible that an effect of hormones on the immune system may be via FSH rather than oestrogen itself, the effect of this hormone was studied on cytokine mRNA expression by PBMC obtained from normal male and female controls. PBMC were cultured for 24 hours with a range of FSH concentrations and the expression of IL-1β, TNF-α, IL-6, TGF-β and prolactin mRNA studied.

The sex hormones have been reported to affect immune function although the mechanism is still unclear (see Introduction, Section 1.6). Cellular proliferation in response to exogenous stimuli is the basis of cell-mediated immunity. Therefore, PBMC from controls and RA patients were incubated for 72 hours with PHA, an anti-CD3 mAb, IL-2 or a combination of the latter two stimuli, alone or with 17β-oestradiol or testosterone at various concentrations. An IL-2 dysfunction has been reported in RA (Combe *et al.*, 1975; Emery *et al.*, 1984), and the response of rheumatoid PBMC to PHA and anti-CD3 mAb have been reported to be abnormal (van den Brink *et al.*, 1992). Thus, by studying the proliferative response of both control PBMC and those from RA patients to these stimuli, as well as the expression and release of cytokines, in the presence and absence of sex hormones, it was hoped to gain more knowledge into the activity of rheumatoid immune cells and hormonal modulation of immune function.

3.2 METHODS

3.2.1 The Effect of Oestrogen and Testosterone on PBMC Cytokine Production

PBMC were prepared from the peripheral blood obtained from normal male and female controls (Method 2.4.1) and resuspended in phenol red-free RPMI supplemented with 10% (v/v) CS-FCS to a concentration of $1X10^6$ cells/ml. Cells were aliquotted into a 24-well plate at $1X10^6$ cells/well and cultured for 24 hour at 37°C, 5% CO_2/95% air, in the presence of medium alone (basal control), ethanol, at a dilution equivalent to that of the highest hormone concentration (vehicle control), 1 μg/ml LPS or 17α-oestradiol, 17β-oestradiol or testosterone at final concentrations of 10^{-12}-10^{-8} M. Following 24 hours incubation, cells were pelleted by centrifuging the plate at 1200 rpm for 10 minutes and the cell supernatants removed into 1.5 ml eppendorf tubes and stored at

-20°C prior to analysis. The IL-6 or TNF content of the supernatants was determined by bioassay (Method 2.5) with the values obtained spectrophotometrically and extrapolated from the relevant standard curve to give U/ml IL-6 or TNF, as described (see Methods 2.5.1 and 2.5.2).

3.2.2 The Effect of Oestrogen on Control and RA PBMC Cytokine mRNA Expression

PBMC were prepared from the peripheral blood obtained from male and female controls and RA patients (Method 2.4.1) and resuspended in either phenol red-free RPMI supplemented with 10% (v/v) CS-FCS or serum-free RPMI to a concentration of $1X10^6$ cells/ml. Cells were aliquotted into 5 cm^2 petri dishes at $10X10^6$ cells/dish and cultured at 37°C, 5% CO_2/95% air. In all experiments untreated cells were incubated with a vehicle control, consisting of ethanol at a dilution equivalent to that of the highest hormone concentration and treated cells consisted of those incubated with 17β-oestradiol at concentrations between 10^{-13} and 10^{-7} M. Additionally, in one experiment, the inactive enantiomer 17α-oestradiol was included at 10^{-9} M, and in two experiments, LPS was included as a positive control, at a final concentration of 1 μg/ml.

Routinely, cells were harvested at 24 hours. In one experiment cells were harvested at 6 and 24 hours and a time course was constructed with time points at 7, 12, 24, 48 or 72 hours. To harvest cells the non-adherent cells were first removed into centrifuge tubes and the adherent cells then dislodged from the surface of the petri dish with a cell-scraper, using ice-cold PBS to facilitate the process. Both cell populations were combined and pelleted by centrifuging at 1000 rpm for 10 minutes. Cell pellets were lysed in Solution D (see Method 2.8.1) and stored at 4°C for up to 1 week. RNA was prepared using the guanidinium-isothiocyanate method (Method 2.8.1) and quantified (Method 2.8.2) prior to either Northern blotting (Method 2.8.5) or dot-blotting (Method 2.8.4). Cytokine cDNA probes were prepared (Method 2.8.7) and labelled (Method 2.8.8) before hybridising to the Northern blot and dot-blot filters (Method 2.8.9) which were then autoradiographed. Scanning densitometry was used to compare bands or dots for alteration of mRNA expression. Filters were stripped (see Method 2.8.9) and stored at 4°C between hybridisations.

3.2.3 The Effect of FSH on PBMC Cytokine mRNA Expression

PBMC were prepared from the peripheral blood obtained from normal male and female controls (Method 2.4.1) and resuspended in serum-free RPMI to a concentration of $1X10^6$ cells/ml. Cells were aliquotted into 5 cm^2 petri dishes at $10X10^6$ cells/dish and cultured at 37°C, 5% CO_2/95% air. Untreated cells were incubated with a vehicle control, consisting of ethanol at a dilution equivalent to that of the highest hormone concentration, and treated cells consisted of those incubated with FSH at concentrations between 10^{-10} and 10^{-7} M. After 24 hours, cells were harvested, the RNA extracted and analysed by dot-blotting, and the filters hybridised to cytokine cDNA probes as described (Section 3.2.2).

3.2.4 The Effect of Testosterone and Oestrogen on Control and RA PBMC Proliferation

PBMC were prepared from the peripheral blood obtained from male and female controls and RA patients (Method 2.4.1) and resuspended in phenol red-free RPMI supplemented with 10% (v/v) CS-FCS to a concentration of $1X10^6$ cells/ml (Method 2.6). Cells were cultured with either a vehicle control, consisting of ethanol at a dilution equivalent to that of the highest hormone concentration, 10^{-9} M 17β-oestradiol or 10^{-9} M testosterone, and either medium alone, an anti-CD3 mAb (αCD3) at a 1/100 dilution, 1μg/ml PHA, 500U/ml rhIL-2 or a combination of αCD3 and rhIL-2. After 72 hours, cells were pulsed, harvested and counted (Method 2.6). Results were analysed as counts (CPM) obtained in the absence of oestrogen or testosterone, or a comparison was made between counts obtained with and without the addition of either hormone.

3.3 RESULTS

3.3.1 The Effect of Oestrogen and Testosterone on PBMC Cytokine Production

The effect of oestrogen and testosterone on cytokine production by PBMC from normal male and female controls was assessed by bioassay in terms of IL-6 and TNF production (Method 3.2.1). The general pattern of IL-6 or TNF release was the same for male and female PBMC, and no obvious correlation was found between oral contraceptive use, menopausal status or stage of menstrus and cytokine production, in

the absence or presence of sex hormones. Therefore, results obtained for male and female PBMC were combined, with the data from five separate IL-6 bioassays and six TNF bioassays pooled and analysed.

As shown in Figure 3.1A and B, 1 μg/ml LPS induced significant PBMC IL-6 and TNF release, with the basal production of IL-6 increased from 12860 to 143200 U/ml and basal TNF production increased from 47 to 1564 U/ml. Cytokine production in the presence of vehicle was not significantly different to that of the basal control (medium alone) with the respective levels of IL-6 being 14800 and 12860 U/ml and that of TNF being 35 and 47 U/ml. 17α-oestradiol, 17β-oestradiol and testosterone, at concentrations ranging from 10^{-12} to 10^{-8} M, had no significant effect on IL-6 or TNF production. There appeared to be some dose-related effect of testosterone with IL-6 and TNF release increased to 38680 and 70 U/ml , respectively, at 10^{-8} M, however this was found to be not significant when analysed using a paired Student's T-test.

3.3.2 The Effect of Oestrogen on PBMC TGF-β mRNA Expression

The effect of oestrogen on PBMC cytokine mRNA expression was initially studied by Northern blot analysis, as demonstrated in Figure 3.2, using a TGF-β cDNA probe. Both experiments (lanes 1-4 and 5-10) utilised PBMC from normal premenopausal female controls. Results demonstrated that the TGF-β cDNA probe annealed to two transcripts of 2.5 and 4.1 kb, corresponding to TGF-β1 and TGF-β2, respectively (Madisen *et al.*, 1988). PBMC were seen to express predominantly the TGF-β1 transcript. In the first experiment there appeared to be a slight enhancement of TGF-β mRNA expression with 10^{-9} M 17β-oestradiol, as can be seen by comparing lanes 1 (control) and 4 (17β-oestradiol). 10^{-9}M was chosen to represent a physiological concentration of oestrogen. In contrast, 17α-oestradiol seemed to depress TGF-β mRNA expression (lane 3) and 1 μg/ml LPS had no effect (lane 2). However, this experiment involved incubating PBMC with the various stimuli for 6 hours in phenol red-free RPMI supplemented with 10% (v/v) CS-FCS, and CS-FCS, although devoid of endogenous steroids, contains growth factors, including TGF-β itself, which may mask any effect with LPS or oestrogen. Indeed, the basal level of TGF-β mRNA expression in RPMI containing CS-FCS (lane 1) was seen to be considerably higher than that in serum-free medium (lane 5) at 6 hours, and this level had declined further at 24 hours (lane 8). Cells cultured in the serum-free medium appeared to demonstrate a considerable upregulation of TGF-β mRNA expression with 10^{-9} M 17β-oestradiol at 24 hours (lane 10), whereas the same concentration of 17α-oestradiol had little effect at

6 (lane 6) or 24 hours (lane 9). Therefore, serum-free medium was used in all subsequent experiments studying hormonal modulation of PBMC cytokine mRNA expression.

As a result of a problem with the NaOH fixation of the filter prior to hybridising with the TGF-β probe, further hybridisations could not be carried out, hence the data were not normalised and the oestrogen effects seen could not be validated. Thus, the experiment was repeated and filters were hybridised to a range of cytokine probes and also to β-actin or GAPDH cDNA, to evaluate whether the results observed were genuine, or simply due to loading inaccuracies.

3.3.3 The Effect of Varying Incubation Times with Oestrogen on PBMC Cytokine mRNA Expression

Based on the preliminary results obtained with oestrogen, as described in Section 3.3.2, a time-course was carried out by incubating PBMC from a normal premenopausal female control with either medium alone or 10^{-9} M 17β-oestradiol, and assessing the mRNA expression at various time points by dot-blotting. Results in Figure 3.3D illustrate that 17β-oestradiol had no effect on the expression of TGF-β at either 7, 12, 24, 48 or 72 hours. The filter was reprobed for IL-1β (A), IL-6 (B), TNF-α (C) and prolactin (E), all of which show the same pattern of mRNA expression, with oestrogen treatment of the cells being ineffective throughout. The filter was normalised by probing for the house-keeping gene, β-actin (Figure 3.3E inset), which showed equal loading.

3.3.4 The Effect of a Range of Oestrogen Concentrations on PBMC Cytokine mRNA Expression

It was considered that the negative results obtained from the time-course experiment may have been due to the use of an inadequate concentration of 17β-oestradiol, therefore a wider range of concentrations was tested. PBMC from a normal premenopausal female control were cultured for 24 hours with 10^{-7}-10^{-13} M 17β-oestradiol, the results of which are shown in Figure 3.4. Hybridisation to IL-1β (A), IL-6 (B), TNF-α (C) and TGF-β (D) cDNA probes demonstrated identical patterns of mRNA expression, with an apparent potentiation at 10^{-10} and 10^{-13} M 17β-oestradiol (lanes 5 and 8, respectively). However, the results with β-actin (Figure 3.4D inset) also

showed the same pattern of mRNA expression indicating that this was due to unequal loading and not to an effect of oestrogen on the PBMC.

3.3.5 The Effect of Oestrogen on Control and RA PBMC Cytokine mRNA Expression

Previous experiments failed to demonstrate an oestrogen effect using a range of hormone concentrations (Figure 3.4) and various incubation times (Figure 3.3). However, both experiments used PBMC from normal premenopausal female controls, therefore PBMC from a wider spectrum of controls and also RA patients were examined. Cells were incubated with 17β-oestradiol at concentrations of 10^{-8}-10^{-12} M and the RNA analysed by dot-blotting and hybridising the filter to a range of cytokine cDNA probes. A GAPDH cDNA probe was used to normalise the data as it has been reported that β-actin can be induced by oestrogen (Hsu & Frankel, 1987). However, as Figure 3.6 illustrates, hybridisation to GAPDH and β-actin cDNA probes produced identical patterns of expression (E inset). Figures 3.5 and 3.6 illustrate the results obtained with PBMC from pre- and peri-menopausal female and male controls, whereas Figures 3.7 and 3.8 illustrate the results with PBMC from pre-, peri- and postmenopausal female and male RA patients. There is no evidence of an oestrogen effect on the mRNA expression of any cytokine tested, although the cells were responsive in culture as 1 μg/ml LPS induced an upregulation of both IL-6 (figure 3.5B, lane 2) and TNF-α (Figure 3.5C, lane 2) mRNA expression at 6 hours. However, IL-1β (A), TGF-β (D) and prolactin (E) expression remained unchanged in the presence of LPS. To further assess the ability of PBMC maintained in serum-free medium to respond to an exogenous stimulus, proliferation to ConA was studied, and found to be comparable to that obtained with cells cultured in medium supplemented with serum (results not shown).

3.3.6 The Effect of FSH on PBMC Cytokine mRNA Expression

PBMC from a normal premenopausal female control were cultured for 24 hours with FSH at concentrations 10^{-7}-10^{-10} M. Total cell RNA was analysed by dot-blotting and hybridising to IL-1β, IL-6, TNF-α, TGF-β and prolactin cDNA probes. As shown in Figure 3.9, FSH had no effect on the expression of any of the cytokines examined.

3.3.7 The Effect of Oestrogen and Testosterone on Control and RA PBMC Proliferation

The effect of 10^{-9} M oestrogen and testosterone on the proliferation of PBMC obtained from a range of RA patients and controls to various stimuli was assessed. There was considerable individual variation in the response to the stimuli tested, thus each individual was included as a separate data point. Initially proliferation to PHA, an anti-CD3 mAb (αCD3), IL-2 or a combination of αCD3 and IL-2 was compared for control and RA PBMC, in the absence of oestrogen or testosterone (Figure 3.10). An unpaired Student's T-test was employed to compare the response of control and RA cells and no statistical difference was found. In addition, neither 17β-oestradiol nor testosterone had any effect on the response of control or RA PBMC to the various stimuli, as shown in Figure 3.11. Basal proliferation increased slightly in the presence of the two hormones, from 584 to 1041 and 1173 CPM with oestrogen and testosterone, respectively, for control PBMC, and from 551 to 618 and 730 CPM with oestrogen and testosterone, respectively, for RA PBMC. However, this was not significant when analysed using a paired Student's T-test (results not shown).

3.4 SUMMARY

The results showed that there was no effect of oestrogen or testosterone, at any of the concentrations tested, on control PBMC IL-6 or TNF-α production. There was also no effect of oestrogen or FSH on control or RA PBMC IL-1β, IL-6, TNF-α, TGF-β or prolactin mRNA expression, using a range of hormone concentrations and various incubation times. The cells were shown to be responsive in culture by the addition of a positive control (LPS). The conclusion of experiments investigating the effects of oestrogen and testosterone on control and RA PBMC proliferation was that neither of the sex hormones affected PBMC response to the stimuli included, and there was no difference in the response of PBMC from controls and RA patients to the stimuli when studied in the absence of hormones.

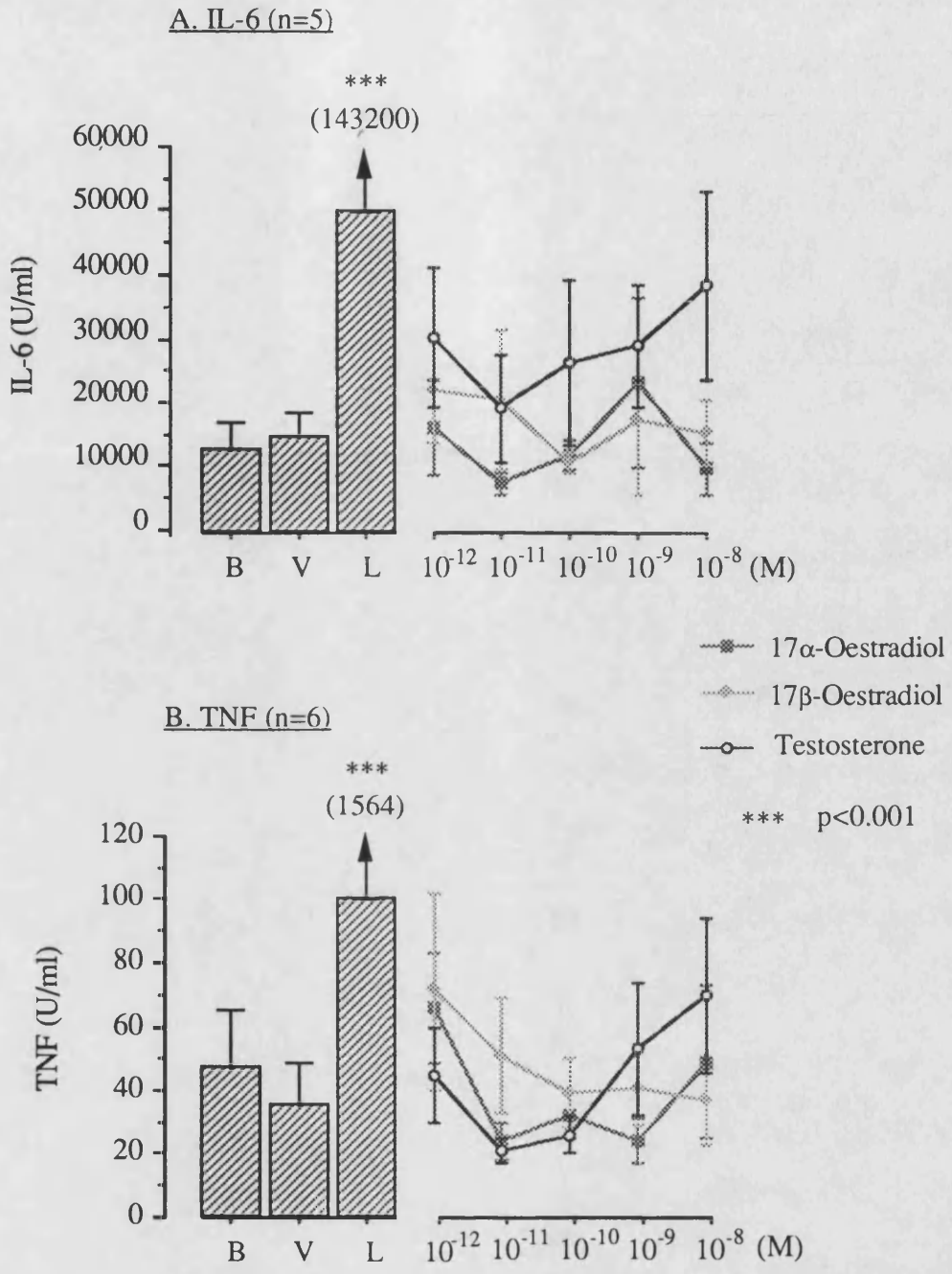


Figure 3.1 The effect of oestrogen and testosterone on PBMC cytokine production

PBMC were obtained from normal male and female controls and cultured for 24 hours in medium alone (basal, B), 0.01% (v/v) ethanol (vehicle, V), 1 μg/ml LPS (L) or 17α-oestradiol, 17β-oestradiol or testosterone at various concentrations. Supernatants were harvested and assayed for (A) IL-6 or (B) TNF content. Results are expressed as U/ml IL-6 or TNF with the data from five or six separate experiments pooled to give mean ± SEM, and analysed using a paired Student's T-test by comparing each treatment group to basal.

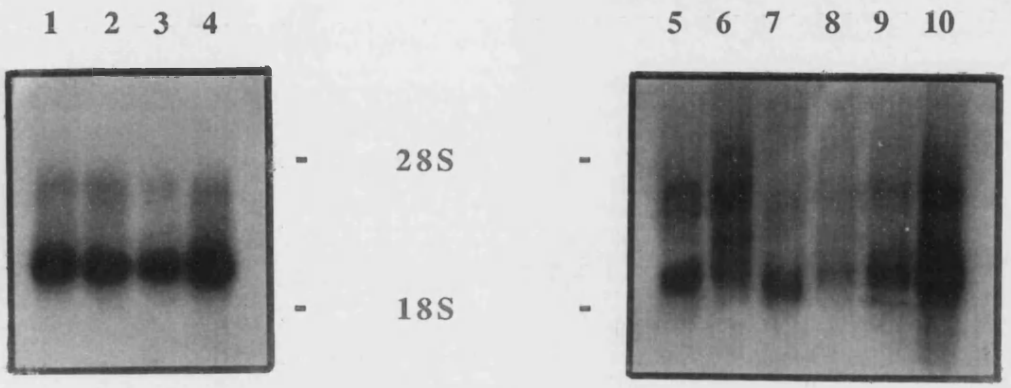
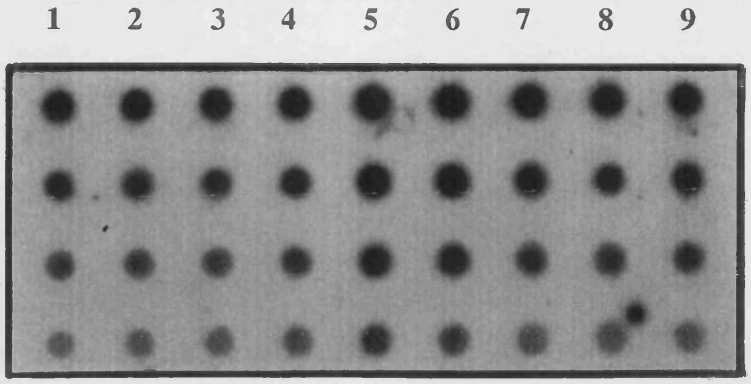


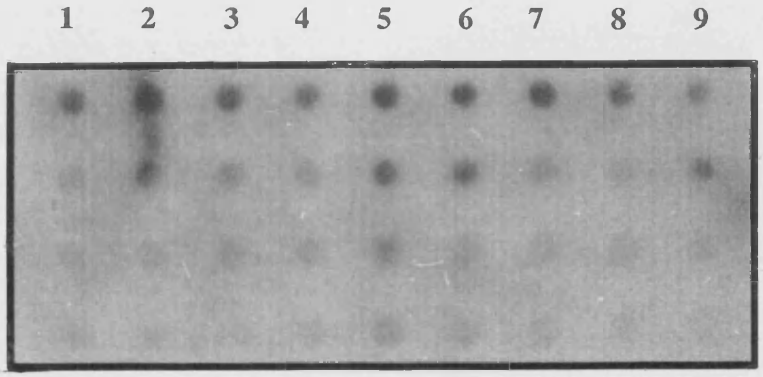
Figure 3.2 The effect of oestrogen on PBMC TGF-β mRNA expression

PBMC were obtained from two normal female controls and cultured in either phenol red-free RPMI supplemented with 10% CS-FCS (lanes 1-4) or in serum-free medium (lanes 5-10). Lanes 1-4 represent cells which were either untreated or treated with 1 μg/ml LPS, 10^{-9} M 17α-oestradiol or 17β-oestradiol, respectively, for 6 hours. Lanes 5-7 and 8-10 represent cells which were untreated or treated with 10^{-9} M 17α-oestradiol or 17β-oestradiol, respectively, for 6 (lanes 5-7) or 24 (lanes 8-10) hours. 9 μg total RNA was loaded onto each lane and analysed by Northern blotting. The filter was hybridised with a TGF-β cDNA probe and autoradiographed.

A. IL-1β



B. IL-6



C. TNF-α

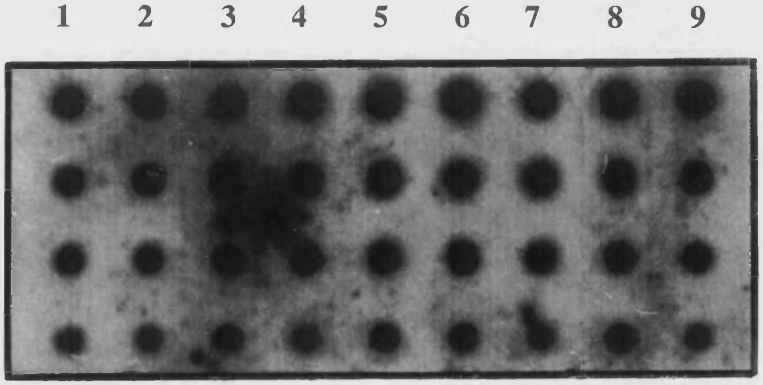


Figure 3.3 Continued...

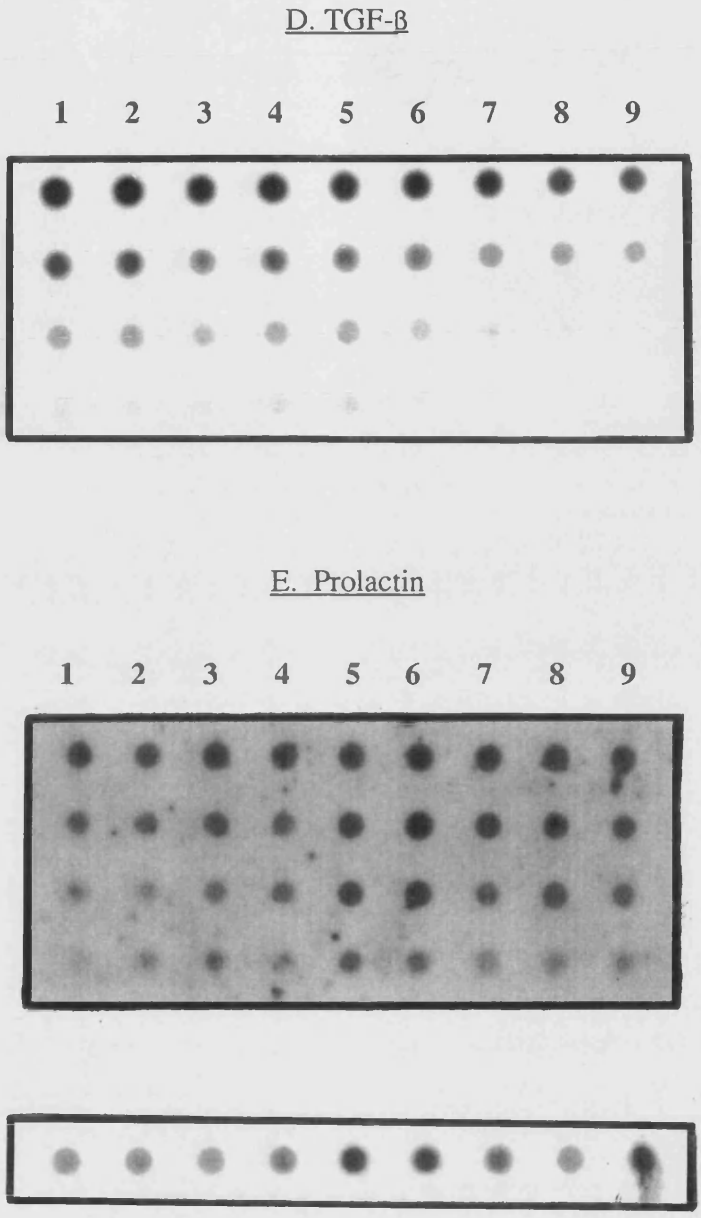
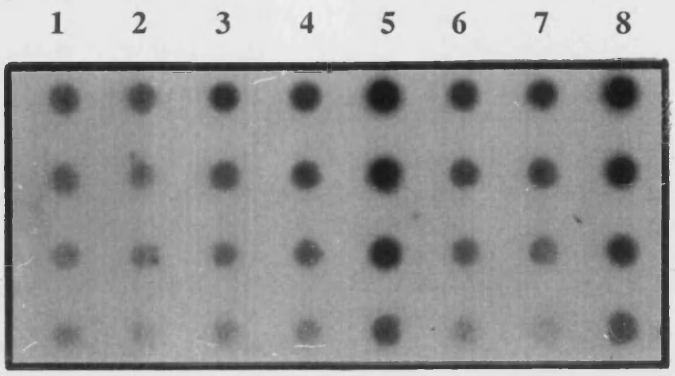


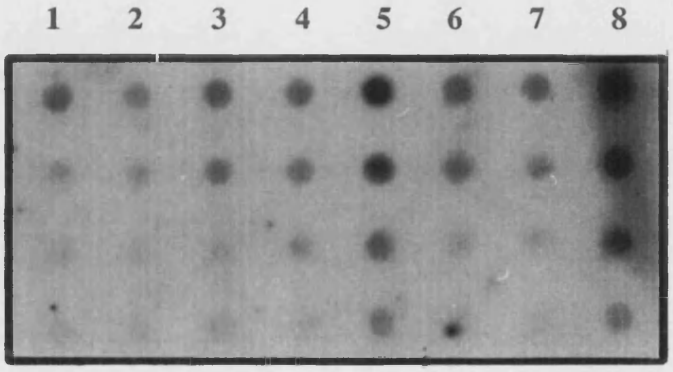
Figure 3.3 The effect of varying incubation times with oestrogen on PBMC cytokine mRNA expression

PBMC were obtained from a normal female control and cultured with medium alone or 10^{-9} M 17β-oestradiol for varying periods of time. Lanes 1, 3, 6 and 8 represent untreated cells at 7, 12, 48 and 72 hours, respectively. Lanes 2, 4, 5, 7 and 9 represent oestrogen-treated cells at 7, 12, 24, 48 and 72 hours, respectively. Total RNA was analysed by dot-blotting and the filter was then hybridised to a range of cytokine cDNA probes and autoradiographed: (A) IL-1β; (B) IL-6; (C) TNF-α; (D) TGF-β and (E) prolactin. All samples were normalised with a β-actin cDNA probe as shown in (E) inset.

A. IL-1β



B. IL-6



C. TNF-α

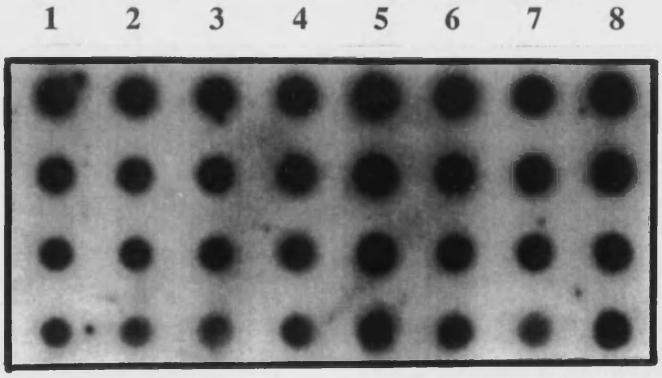


Figure 3.4 Continued...

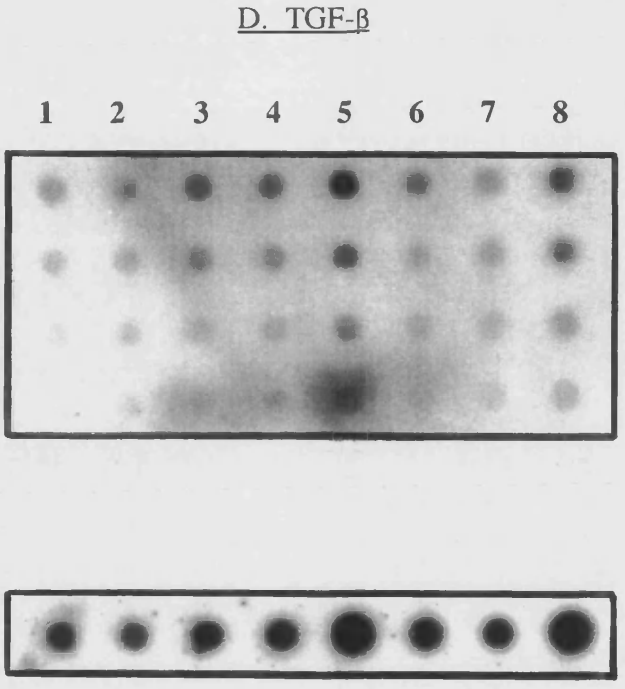
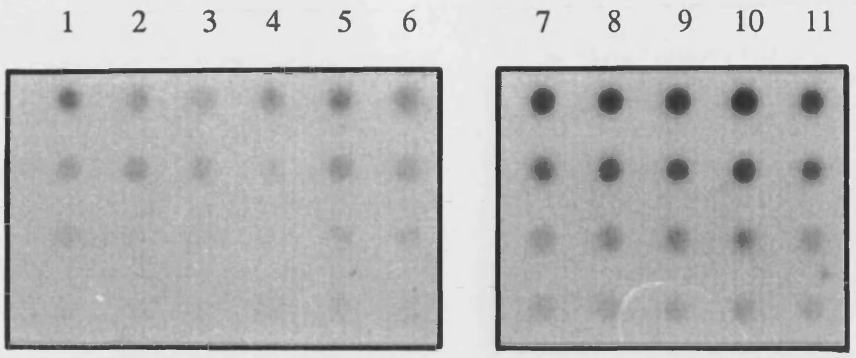


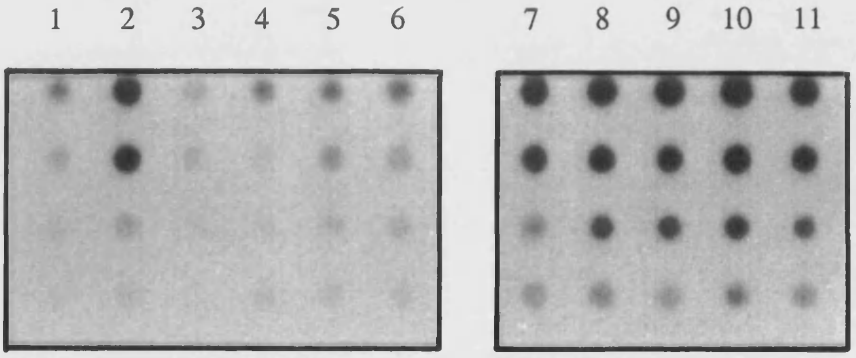
Figure 3.4 The effect of a range of oestrogen concentrations on PBMC cytokine mRNA expression

PBMC were obtained from a normal female control and cultured for 24 hours with varying concentrations of 17β-oestradiol or with medium alone. Lane 1 represents untreated cells and lanes 2-8 represent cells treated with 10^{-7}-10^{-13} M 17β-oestradiol, respectively. Total RNA was analysed by dot-blotting and the filter was then hybridised to a range of cytokine cDNA probes and autoradiographed: (A) IL-1β; (B) IL-6; (C) TNF-α and (D) TGF-β. All samples were normalised with a β-actin cDNA probe as shown in (D) inset.

A. IL-1ß



B. IL-6



C. TNF-α

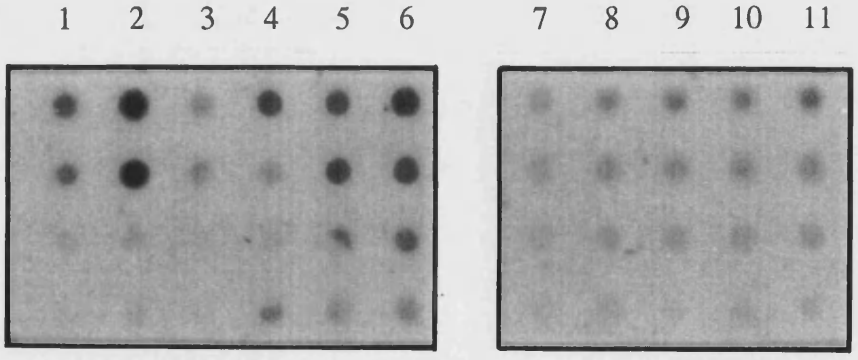


Figure 3.5. Continued...

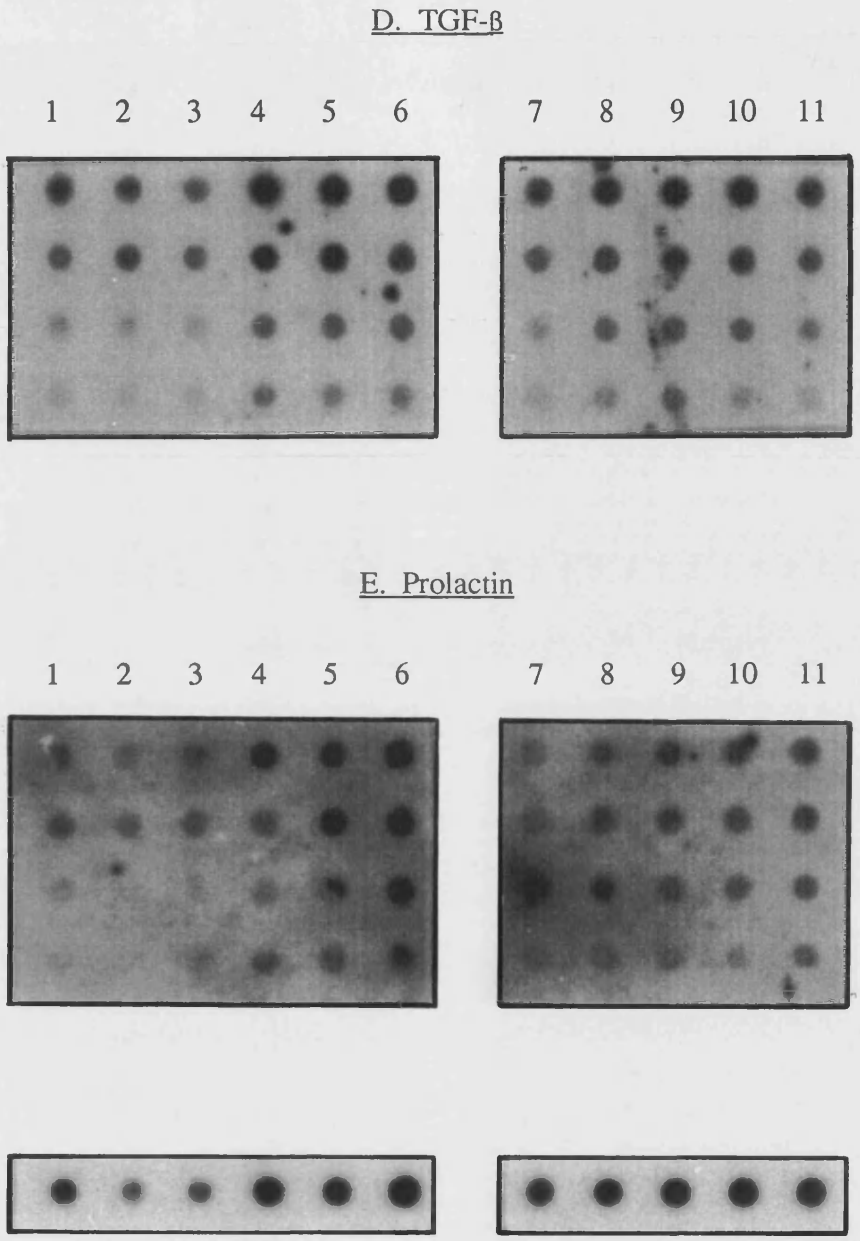
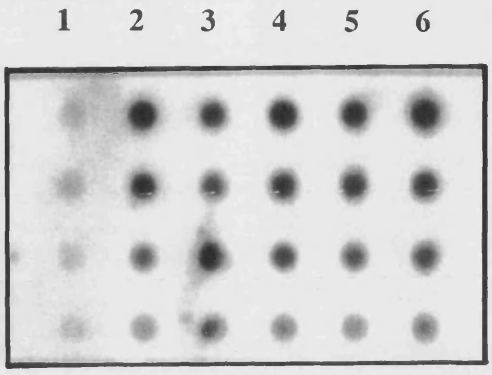


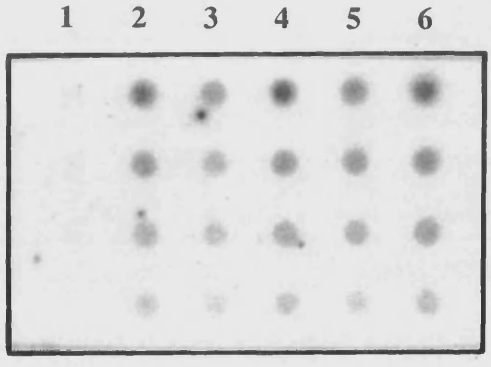
Figure 3.5 Oestrogen modulation of cytokine mRNA expression by PBMC from pre- and perimenopausal female controls

PBMC were obtained from a normal premenopausal (lanes 7-11) or perimenopausal (lanes 1-6) female controls and cultured for 24 hours without stimulus (lanes 1 and 7) or in the presence of 10^{-8}-10^{-12}M (lanes 3-6) or 10^{-9}-10^{-12} M (lanes 8-11) 17β-oestradiol, or for 6 hours with 1 μg/ml LPS (lane 2). Total RNA was analysed by dot-blotting and the filter was then hybridised to a range of cytokine cDNA probes and autoradiographed: (A) IL-1β; (B) IL-6; (C) TNF-α; (D) TGF-β and (E) prolactin. All samples were normalised with a GAPDH cDNA probe as shown in (E) inset.

A. IL-1β



B. IL-6



C. TNF-α

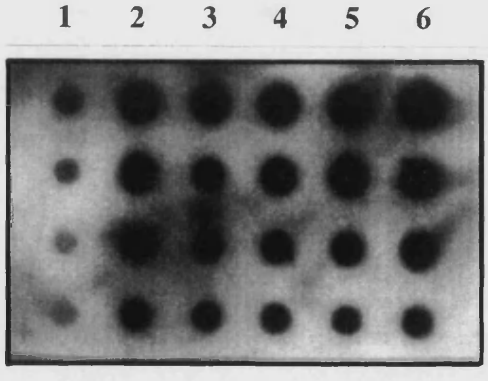
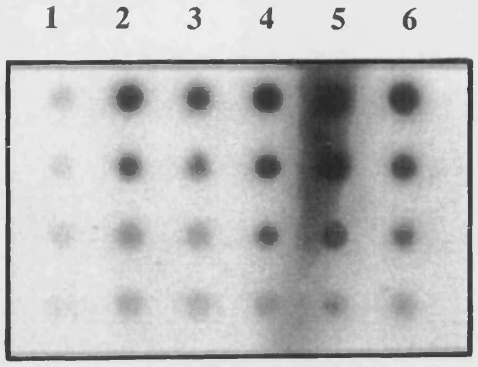


Figure 3.6 Continued...

D. TGF-β



E. Prolactin

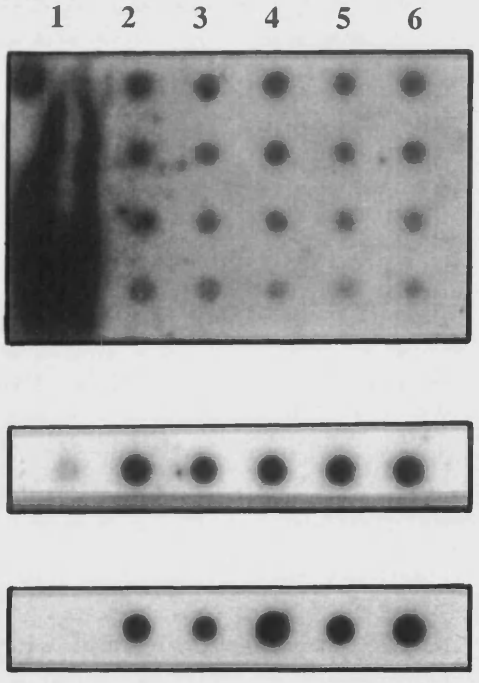
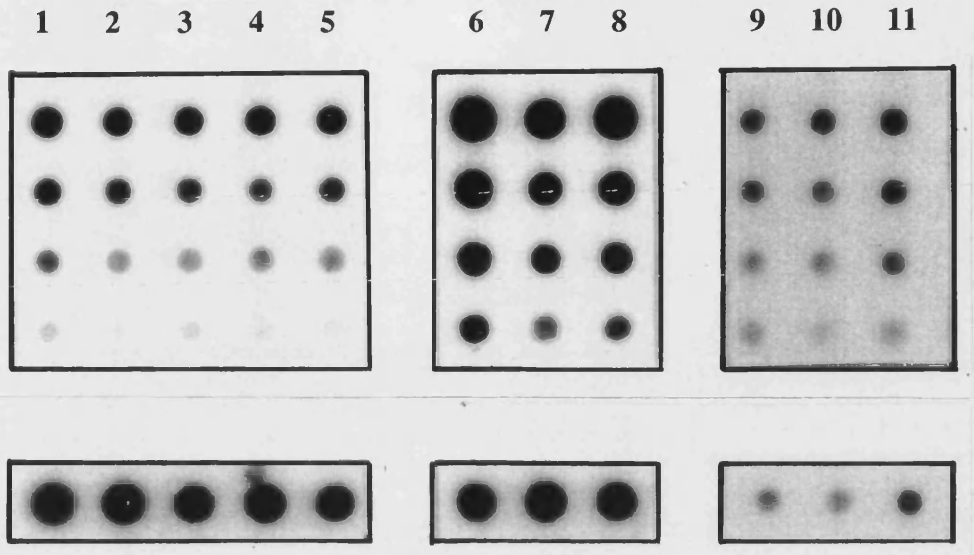


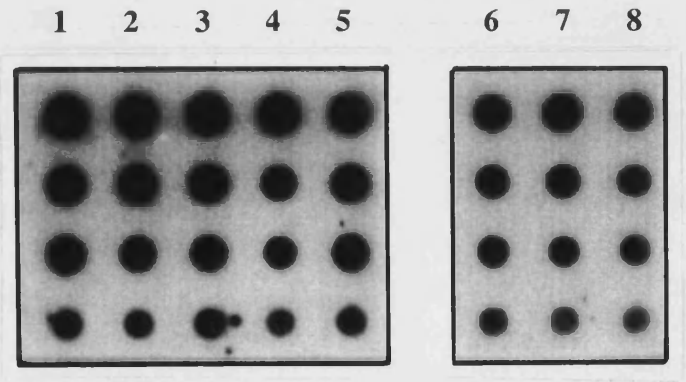
Figure 3.6 Oestrogen modulation of cytokine mRNA expression by PBMC from a male control

PBMC were obtained from a normal male control and cultured for 24 hours without stimulus (lane 1) or with 10^{-8}-10^{-12} M 17β-oestradiol (lanes 2-6). Total RNA was analysed by dot-blotting and the filter was then hybridised to a range of cytokine cDNA probes and autoradiographed: (A) IL-1β; (B) IL-6; (C) TNF-α; (D) TGF-β and (E) prolactin. All samples were normalised with β-actin and GAPDH cDNA probes as shown in (E) insets.

A. IL-1β



B. IL-6



C. TNF-α

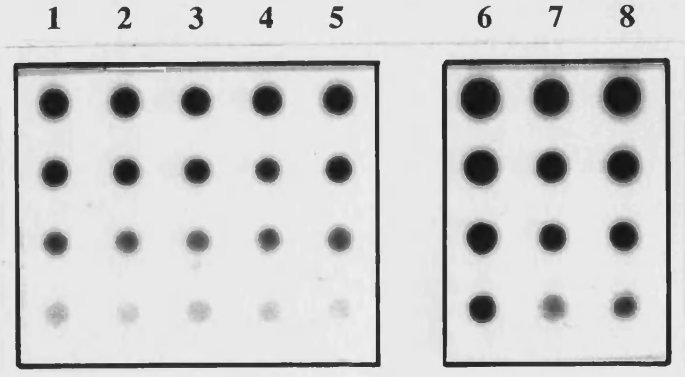
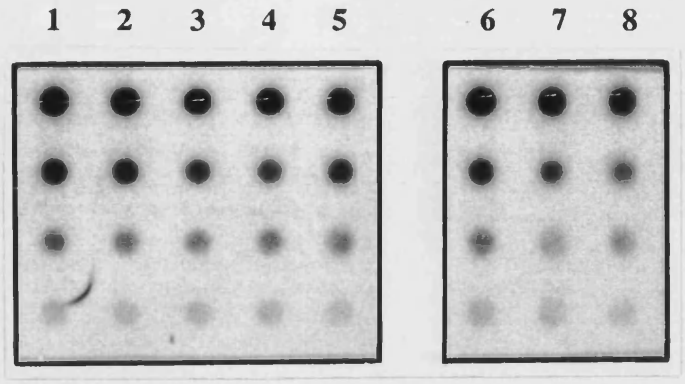


Figure 3.7 Continued...

D. TGF-β



E. Prolactin

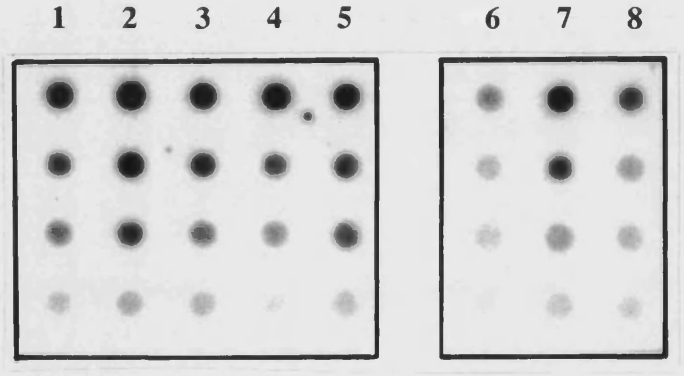
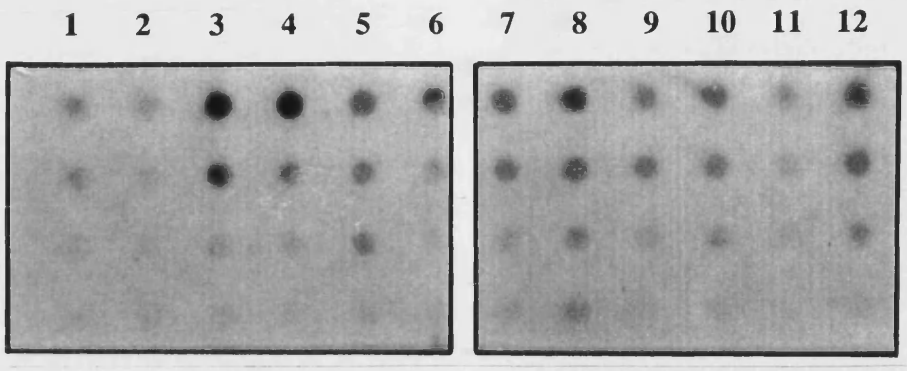


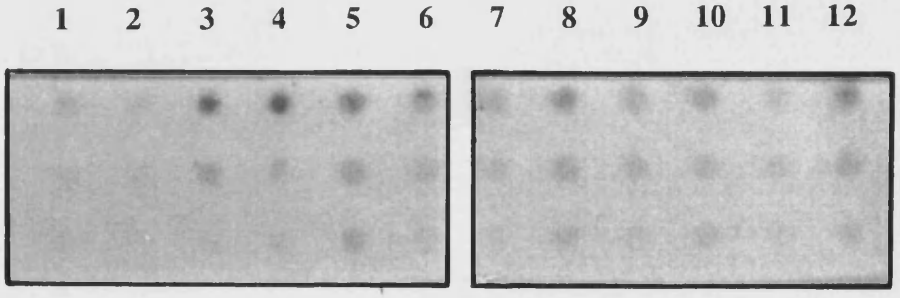
Figure 3.7 Oestrogen modulation of cytokine expression by PBMC from pre- and postmenopausal female and male RA patients

PBMC were obtained from a premenopausal (lanes 1-5) and postmenopausal (lanes 9-11) female RA patient and from a male RA patient (lanes 6-8). Cells were cultured for 24 hours without stimulus (lanes 1, 6 and 9) or with 10^{-8}-10^{-11} M (lanes 2-5) or 10^{-9} and 10^{-11} M 17β-oestradiol (lanes 7 and 8 and lanes 10 and 11, respectively). Total RNA was analysed by dot-blotting and the filter was then hybridised to a range of cytokine cDNA probes and autoradiographed: (A) IL-1β; (B) IL-6; (C) TNF-α; (D) TGF-β and (E) prolactin. All samples were normalised with a GAPDH cDNA probe as shown in (A) inset.

A. IL-1β



B. IL-6



C. TNF-α

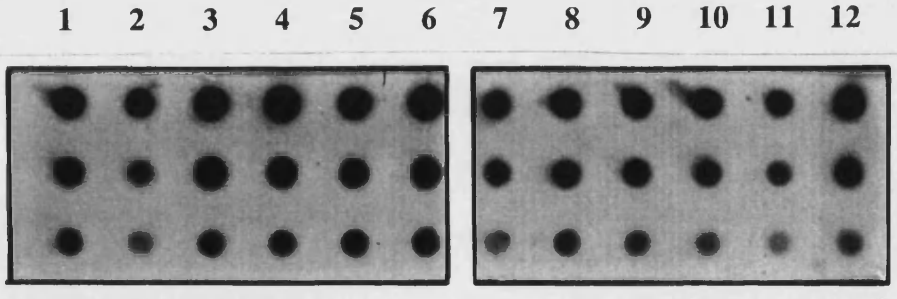


Figure 3.8 Continued...

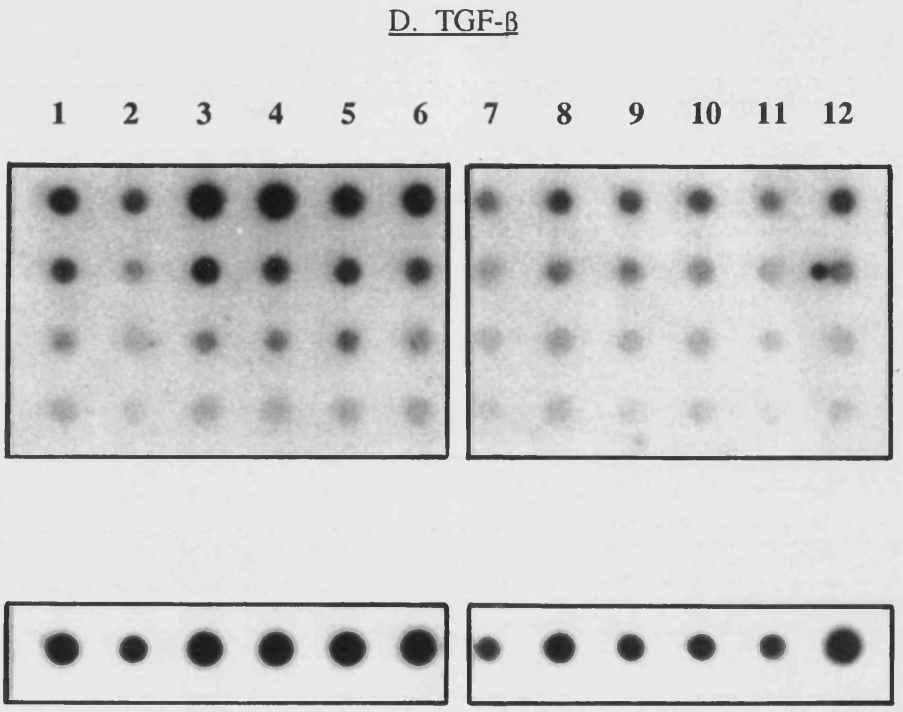
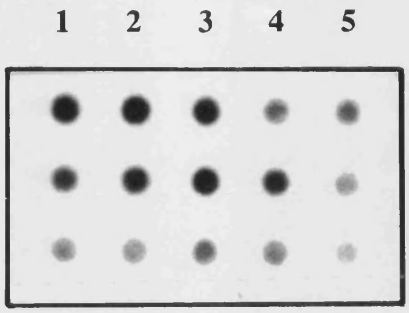


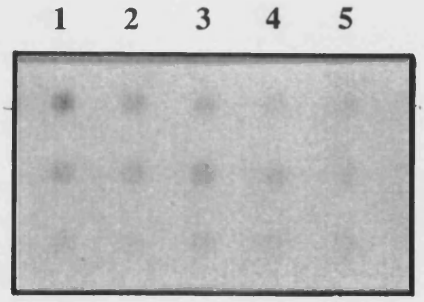
Figure 3.8 Oestrogen modulation of cytokine expression by PBMC from perimenopausal female and male RA patients

PBMC were obtained from a perimenopausal female RA patient (lanes 7-12) and from a male RA patient (lanes 1-6). Cells were cultured for 24 hours without stimulus (lanes 1 and 7) or with 10^{-8}-10^{-12} M 17β-oestradiol (lanes 2-6 and 8-12). Total RNA was analysed by dot-blotting and the filter was then hybridised to a range of cytokine cDNA probes and autoradiographed: (A) IL-1β; (B) IL-6; (C) TNF-α and (D) TGF-β. All samples were normalised with a GAPDH cDNA probe as shown in (D) inset.

A. IL-1β



B. IL-6



C. TNF-α

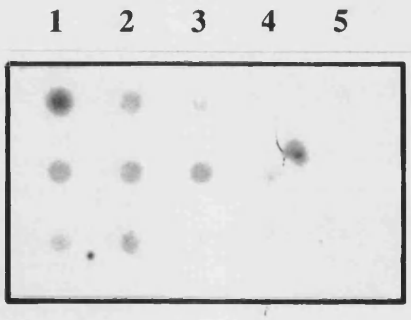


Figure 3.9 Continued...

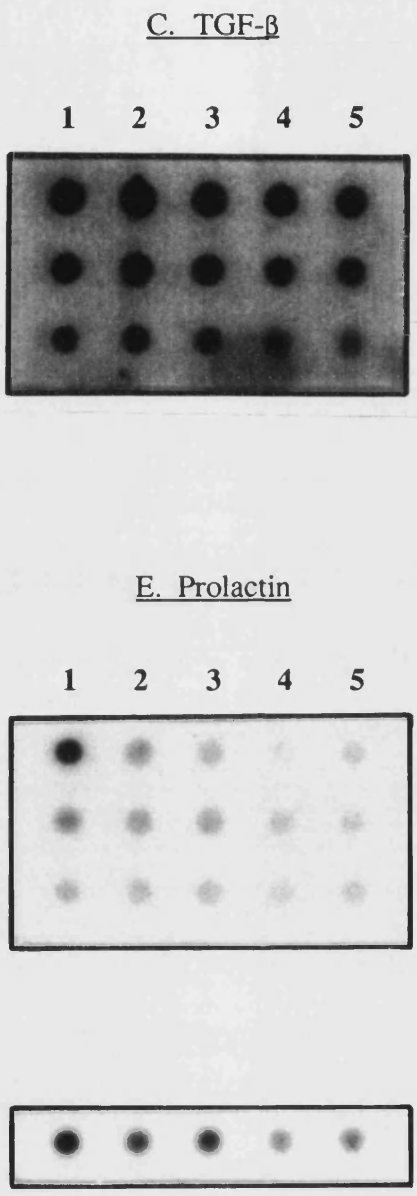


Figure 3.9 The effect of FSH on PBMC cytokine mRNA expression

PBMC were obtained from a normal female control and cultured for 24 hours in the absence of stimulus (lane 1) or with 10^{-7}-10^{-10} M FSH (lanes 2-5). Total RNA was analysed by dot-blotting and the filter was then hybridised to a range of cytokine cDNA probes and autoradiographed: (A) IL-1β; (B) IL-6; (C) TNF-α; (D) TGF-β and (E) prolactin. All samples were normalised with a GAPDH cDNA probe as shown in (E) inset.

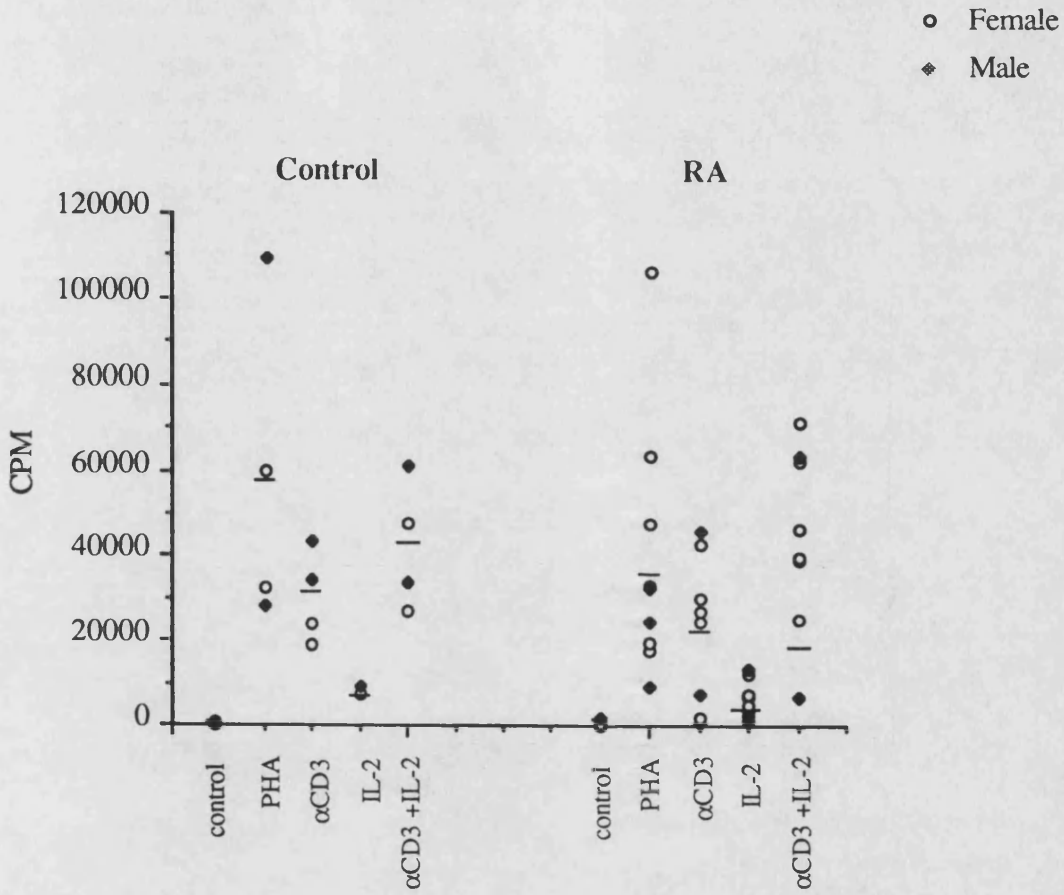
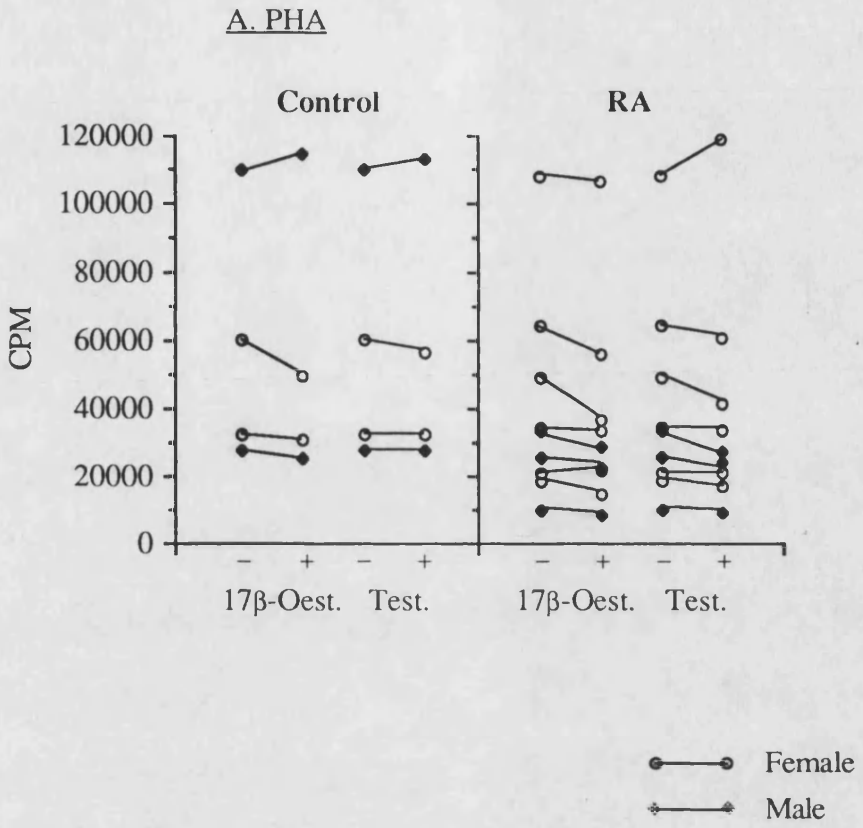


Figure 3.10 Proliferation of PBMC from controls and RA patients

PBMC were obtained from one pre- and one postmenopausal female and two male controls, and from three pre- and three postmenopausal female and three male RA patients. Cells were cultured with medium alone (control), 1 μg/ml PHA (PHA), 1/100 dilution anti-CD3 mAb (αCD3), 500 U/ml rhIL-2 (IL-2) or αCD3 in combination with rhIL-2 (αCD3 + IL-2). After 72 hours cells were pulsed with [^{3}H]thymidine for 4 hours, harvested and counted, and proliferation assessed. Results are expressed as CPM, with the data for each individual included as a separate point, and analysed using an unpaired Student's T-test by comparing control to RA for each treatment.



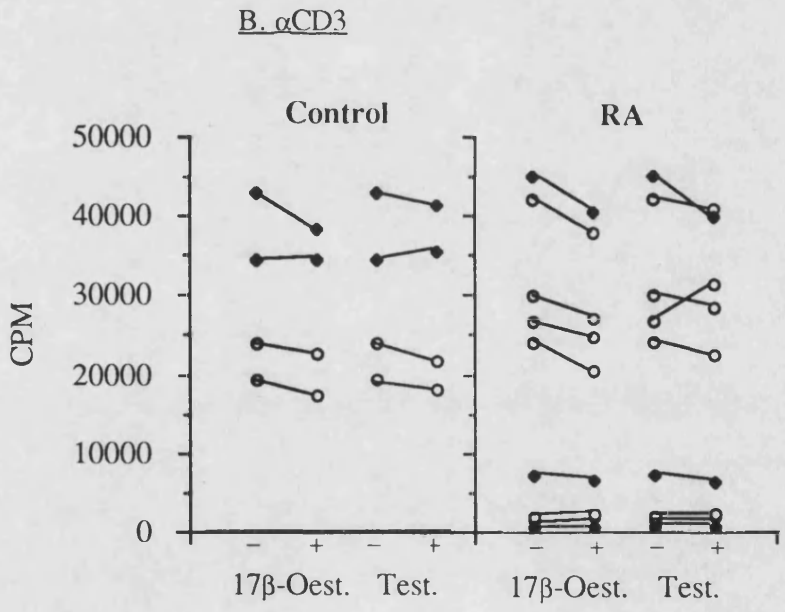


Figure 3.11. Continued...

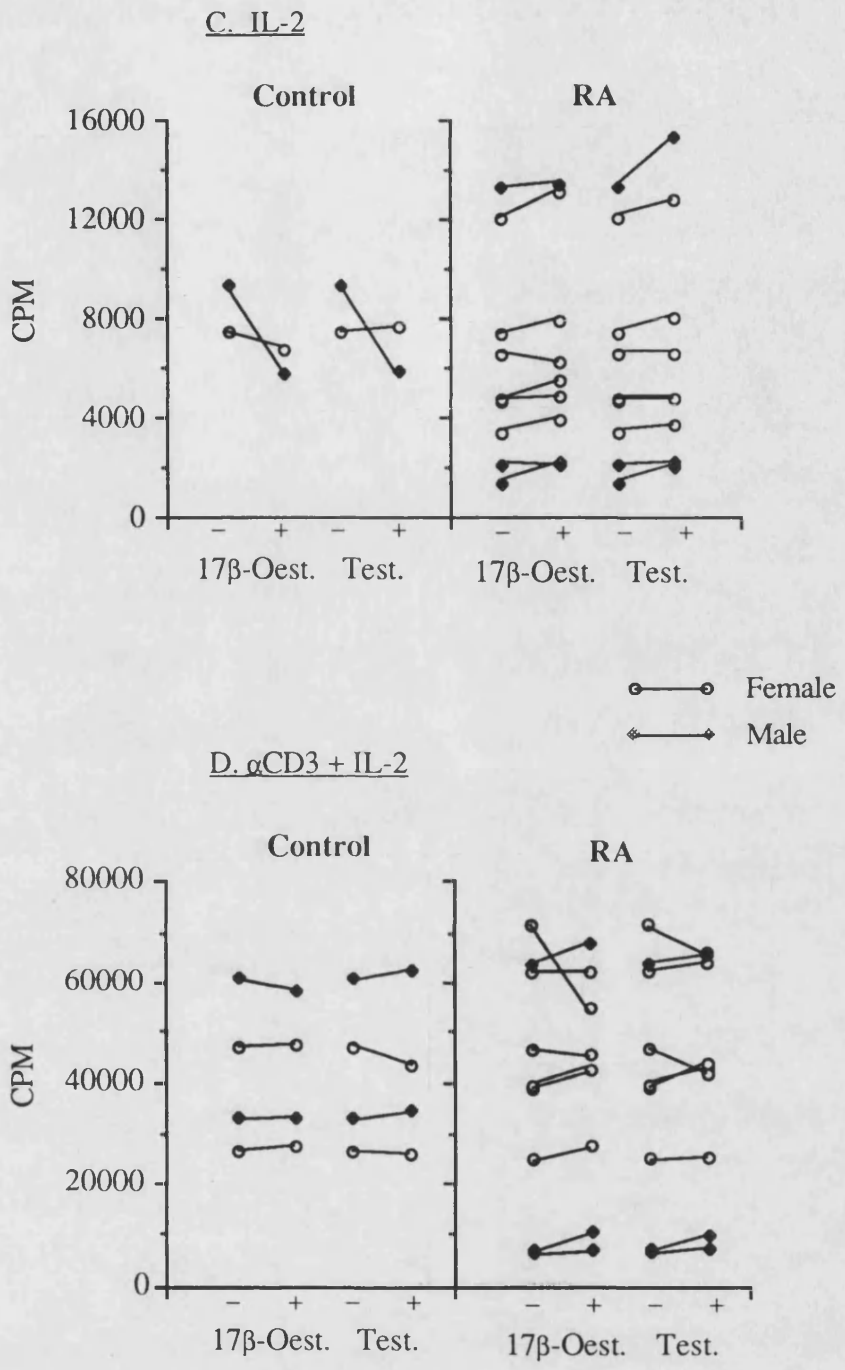


Figure 3.11 The effect of oestrogen and testosterone on control and RA PBMC proliferation

PBMC were obtained from one pre- and one postmenopausal female and two male controls, and from three pre- and three postmenopausal female and three male RA patients. Cells were cultured with medium alone (control), 1 μg/ml PHA (PHA), 1/100 dilution anti-CD3 mAb (αCD3), 500 U/ml rhIL-2 (IL-2) or αCD3 in combination with rhIL-2 (αCD3 + IL-2), in the presence or absence of 10^{-9} M 17β-oestradiol or 10^{-9} M testosterone. After 72 hours cells were pulsed with [^{3}H]thymidine for 4 hours, harvested and counted, and proliferation assessed. Results are expressed as CPM, with the paired samples representing cells cultured in the absence (-) and presence (+) of hormone, for each individual.

CHAPTER FOUR

OESTROGEN RECEPTOR

4.1 INTRODUCTION

Chapter Three discussed the findings of experiments investigating the effect of sex hormones, in particular oestrogen, on PBMC cytokine production, at the protein and transcriptional level, and on PBMC proliferation. The results obtained were negative, which questioned the activity of the 17β-oestradiol used and the expression of ER by the cells. There are few reports of ER expression by cells of the immune system, and those studies cited in the literature have concentrated on the detection of ER protein rather than mRNA expression. For example, oestrogen binding sites were detected on human leukaemic cells (Danel *et al.*, 1981) and later on human PBMC (Danel *et al.*, 1983), in particular it appeared that the CD8+ T cell population was ER positive with CD4+ cells being negative (Cohen *et al.*, 1983; Stimson, 1988). ERs have also been demonstrated on a human monocytic cell line and on rat peritoneal macrophages (Gulshan *et al.*, 1990). However, a recent review doubted previous findings as attempts to demonstrate the expression of mRNA in T cells were unsuccessful (Lahita, 1990).

Experiments were designed based on the necessity to validate previous negative results. In the first instance the efficacy of 17β-oestradiol used in studies reported in Chapter Three was assessed using cell lines known to be either oestrogen responsive or unresponsive, namely the breast carcinoma cell lines, ZR-75, T-47D and Hs578T. The ZR-75 (Engel *et al.*, 1978) and T-47D (Keydar *et al.*, 1979) cell lines are ER-positive and oestrogen-responsive, whereas the Hs578T cell line is ER-negative and oestrogen-unresponsive (Hackett *et al.*, 1977). The proliferation of the three cell lines was studied in response to 17β-oestradiol and the less active enantiomer, 17α-oestradiol. In addition, the anti-oestrogen, tamoxifen, was used to determine the specificity of cell responses to oestrogen.

Secondly, the expression of ER by PBMC, both from controls and RA patients was assessed in order to attempt to clarify contradictions in the literature. The cell lines discussed above were included as positive and negative controls, and, as the joint is the centre of activity in RA, synovial fibroblasts and sections of RA synovial tissue were also studied. The initial investigations of sex hormone receptors published in the literature involved the use of binding assays using radiolabelled steroid, in particular the dextran-coated charcoal assay. However, this is an indirect measurement and does not allow for the discrimination between heterogeneous cell populations. In addition, contamination of samples with other steroid-binding proteins could bias results. The necessity for more sensitive and specific ER detection methods for breast carcinoma

evaluation led to the development of the immunocytochemical assay (ICA), using specific anti-ER mAbs. However, initial studies carried out as part of this project, with a mAb reported to be directed against human ER, proved unsuccessful, as the antibody reacted non-specifically with all cells tested.

The Abbott ER-ICA kit, which employs the H222 mAb to recognise a portion of the ER molecule separate from the oestrogen-binding site, has provided an efficient and specific ER detection system and is now used routinely for diagnostic purposes (Allred *et al.*, 1990). H222 was one of a range of ER-specific mAbs which enabled the nuclear localisation of the ER to be determined and which hence led to the disproval of the 'two-step' model for oestrogen action (see Introduction, Section 1.2.2) (King & Greene, 1984; Greene & Press, 1986). Applications for the H222 mAb do not seem to be limited to the detection of ER in breast tumour cells and oestrogen-responsive tissues, in that it has been used to detect ER in human osteoclasts (Pensler *et al.*, 1990). Therefore, the ER-ICA kit was employed with ZR-75 and Hs578T cell lines, PBMC, synovial fibroblasts and synovium sections. Flow cytometry provides a means of accurately distinguishing, qualitatively and quantitatively, between cell populations expressing specific antigenic markers, by labelling with fluorescent conjugates. Thus, a modification of the paraformaldehyde-saponin procedure (Sander *et al.*, 1991) was employed, as the ER is present intracellularly, and the above cell types were labelled with the H222 mAb and examined by FACS analysis.

The expression of ER in cells and tissues which are thought of as being 'non-oestrogen responsive', such as cells of the immune system, is hampered by the very low concentration of receptor sites. The use of the H222 mAb for immunocytochemistry and flow cytometry proved to be unsuccessful and therefore a more sensitive means of detecting the limited number of ER was sought. An antibody has been described which coprecipitates radiolabelled ER from human breast tumour and endometrial preparations (Coffer *et al.*, 1985a). The ER-D5 antibody was raised in mice against a partially purified ER preparation from human myometrium and was found to recognise a 29 kD cytoplasmic serine phosphoprotein (p29), which is distinct from the ligand binding unit of ER (Coffer & King, 1988). p29 appears to be closely associated with the ER in that there was a strong correlation between ER-D5 binding and ER sites in a series of human breast tumours (Coffer *et al.*, 1985b), and the antigen has been shown to be at least as effective as ER in predicting the hormone sensitivity of breast tumours (Cano *et al.*, 1986). As p29 is thought to be present at approximately one thousand times the concentration of ER in oestrogen-responsive tissues, and the ER-D5 was successful in detecting ER in human bone-derived cells where the H222 mAb failed (Colston *et al.*,

1989), ER-D5 was used with cells and sections in the APAAP immunocytochemical assay and cells were also examined by FACS analysis.

The use of the antibody ER-D5 to detect p29 antigen may provide a sensitive method in situations where ER protein is below the detection limit of conventional anti-ER antibodies, but the specificity of this antigen has yet to be confirmed. Therefore, as an alternative to studying the expression of ER protein, ER mRNA expression was investigated. Greene *et al.* (1986) published the sequence for human ER cDNA and from this a cDNA probe was designed which hybridised to the 6.2 kb ER mRNA transcript of MCF-7 cells and coded for the synthesis of immunoreactive 65 kD ER. The ER cDNA was used with mRNA from bone cells to detect an ER mRNA transcript of 6-6.2 kb (Eriksen *et al.*, 1988; Komm *et al.*, 1988). Therefore, to analyse ER mRNA expression, total RNA was obtained from PBMC and from the cell lines ZR-75, T-47D and Hs578T, and Northern blotted. The filter was hybridised to an ER cDNA probe and analysed by autoradiography.

In recent years, the PCR has gained precedence as an extremely sensitive method for amplification of a specific cDNA sequence by the use of two primers which flank the sequence of interest. Reverse transcription provides the ability to produce single-stranded cDNA from total RNA, hence the two techniques can be combined, RT-PCR, to enable the detection of small amounts of mRNA. In addition, RT-PCR has been used for the detection of ER mRNA, and has gained application in situations where Northern blot analysis was unsuccessful (Hirata *et al.*, 1992; Liu *et al.*, 1992). Using this information, specific primers were designed based on the human ER cDNA sequence, and RNA from PBMC, synovial fibroblasts and cell lines was reverse transcribed and amplified using the oligonucleotide primer set, in an attempt to detect the presence of ER mRNA.

4.2 METHODS

4.2.1 The Effect of Oestrogen on ZR-75, T-47D and Hs578T Cell Proliferation

Confluent ZR-75, T-47D and Hs578T cells were trypsinised, washed twice in the relevant culture medium and resuspended in phenol red-free RPMI supplemented with 10% (v/v) CS-FCS to a concentration of $1X10^6$ cells/ml (Method 2.6). Cells were cultured with either a vehicle control, consisting of ethanol at a dilution equivalent to

that of the highest hormone concentration, or 10^{-7}-10^{-13} M 17α-oestradiol or 17β-oestradiol, with each test performed in triplicate. After 72 hours, cells were pulsed, harvested and counted (Method 2.6). Results were analysed as average counts (CPM) for each triplicate and the effect of oestrogen on cell proliferation assessed.

4.2.2 The Effect of Tamoxifen on Oestrogen-Induced ZR-75 Cell Proliferation

Confluent ZR-75 cells were trypsinised, washed twice in the relevant culture medium and resuspended in phenol red-free RPMI supplemented with 10% (v/v) CS-FCS to a concentration of $1X10^6$ cells/ml (Method 2.6). Cells were cultured with either a vehicle control, consisting of ethanol at a dilution equivalent to that of the highest hormone concentration, 10^{-9} M 17β-oestradiol, or with tamoxifen alone, at a concentration of 10^{-5} M, or in combination with 10^{-9} M 17β-oestradiol, at concentrations 10^{-11}-10^{-5} M, with each test performed in triplicate. After 72 hours, cells were pulsed, harvested and counted (Method 2.6). Results were analysed as average counts (CPM) for each triplicate and the effect of tamoxifen on basal cell proliferation and oestrogen-induced cell proliferation assessed.

4.2.3 Northern Blot Analysis of ER mRNA Expression

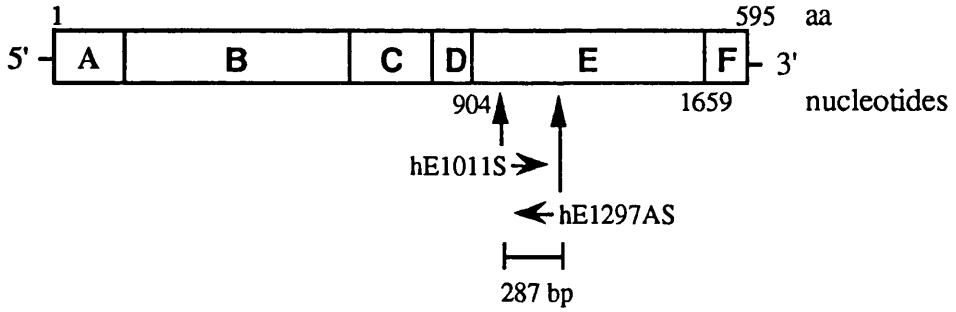
Total cell RNA was prepared from freshly purified PBMC obtained from a normal female control, and from confluent ZR-75, T-47D and Hs578T cells (Method 2.8.1) and was Northern blotted (Method 2.8.5). The ER cDNA probe was prepared from pER35 (Method 2.8.7), labelled (Method 2.8.8) and hybridised to the Northern blot filter. A modified method of hybridisation was used, whereby the filter was added to a prehybridisation solution (5X SSPE, 5X Denhardt's, 0.5% (w/v) SDS, 50% (v/v) formamide, 20 μg/ml salmon sperm DNA) and incubated at 50°C for 5 hours. The labelled ER cDNA probe was then added to the prehybridisation mix and the filter incubated at 42°C overnight. After removing the hybridisation solution containing the probe, the filter was washed at 65°C, twice in 1X SSPE, 0.1% (w/v) SDS for 15 minutes each wash, and twice in 0.1X SSPE, 0.1% (w/v) SDS for 10 minutes each wash, then autoradiographed.

4.2.4 Southern Blot Analysis of ER cDNA

1 µg plasmid DNA containing ER cDNA was subjected to a restriction nuclease digestion to release the ER cDNA insert, and electrophoresed on a 0.8% (w/v) agarose gel with λHE as a marker, and Southern blotted overnight (Method 2.8.6). The filter was hybridised to an ER cDNA probe (Method 2.8.9), prepared and labelled as described (Methods 2.8.7 and 2.8.8), and autoradiographed.

4.2.5 Designing Oligonucleotide Primers for the Amplification of ER cDNA

Hirata *et al.* (1992) reported the amplification of ER cDNA utilising a primer set which flanked the oestrogen binding domain region of rat ER cDNA. Based on this, oligonucleotide primers were designed which corresponded to the same region of the human ER cDNA sequence (Greene *et al.*, 1986) and synthesised by Colin Lazarus (Bristol University, Bristol, UK). The forward primer (hE 1011S) 5'-CAGTGAAGCT TCGATGATGG-3' (nucleotides 1011-1030) and the reverse primer (hE1297AS) 5'-ATGATGTAGCCAGCAGCATG-3' (nucleotides 1297-1278) frame a 287 bp-long region encoding part of the hormone-binding domain of human ER:



4.2.6 Analysis of ER mRNA Expression by RT-PCR

Total cell RNA prepared from a range of cell types including control and RA PBMC, synovial fibroblasts and the cell lines ZR-75, T-47D and Hs578T, was reverse transcribed using either M-MLV or Superscript reverse transcriptase (Method 2.8.10). The single stranded cDNA obtained was amplified during 32 cycles with Taq DNA polymerase and the gene-specific primers hE1011S and hE1297AS by PCR (Method

2.8.11). Each cycle consisted of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1 minute. The annealing (or melting) temperature (T_m) was calculated using the following equation:

$$T_m = 81.5 - 16.6(Log_{10}[Na^+]) + 0.41(\%G+C) - (600/N)$$

The PCR product was analysed by agarose gel electrophoresis.

Taq DNA polymerase from NBL was supplied with a 10X buffer containing 15 mM $MgCl_2$, whereas that obtained from Promega Ltd. or Perkin Elmer Ltd. was supplied with a $MgCl_2$-free buffer, and thus a $MgCl_2$ titration was carried out in order to determine the optimal concentration required for each enzyme. This was achieved by amplifying ZR-75 cell cDNA by PCR, as described above, in a reaction mixture containing 0-6 mM $MgCl_2$, with the concentration giving optimal DNA amplification chosen for use in all subsequent reactions.

4.2.7 Purification of Individual PCR Products by Band-Stab PCR

To purify two separate products occurring as individual bands in the same PCR reaction, a band-stab PCR method was employed (Bjourson & Cooper, 1992). This comprised of electrophoresing the product through a 4% (w/v) NuSieve low melting temperature agarose gel, stained with ethidium bromide, which was then viewed on a UV transilluminator. The appropriate bands were stabbed with a sterile hypodermic needle which was subsequently inserted into a reaction mix containing all PCR components except the DNA (Method 2.9.11). The two products were re-amplified and analysed as before.

4.2.8 Sequencing ER cDNA

ZR-75 cell RNA was reverse transcribed and amplified with the gene specific primers, hE1011S and hE1297AS, designed to flank a 287 bp region of the human ER hormone-binding domain, as described (see Section 4.2.6). The cDNA product was then sequenced, using end-labelled forward and reverse oligonucleotide ER primers (Method 2.8.12).

4.3 RESULTS

4.3.1 The Effect of Oestrogen on ZR-75, T-47D and Hs578T Cell Proliferation

To assess the effectiveness of the active enantiomer, 17β-oestradiol, in comparison to the less active enantiomer, 17α-oestradiol, the proliferation of cell lines reported to be either ER-positive or -negative was studied. Following 72 hours culture with 10^{-13}-10^{-7} M 17α-oestradiol the proliferation of T-47D and Hs578T cells remained unchanged, as shown in Figure 4.1B and C, respectively. 17β-oestradiol had a tendency to suppress the proliferation of these two cell lines, which was significant ($p<0.05$) at 10^{-11} M for T-47D (B) and at 10^{-12} and 10^{-10} M for Hs578T (C). In contrast, the proliferation of ZR-75 cells was increased with concentrations of 17β-oestradiol from 10^{-11} to 10^{-7} M (Figure 4.1A). A maximum was reached at 10^{-8} M 17β-oestradiol of 180% compared to the control, significant at $p<0.001$. With respect to 17α-oestradiol, at concentrations below 10^{-8} M ZR-75 cell proliferation was suppressed, and this inhibition was found to be significant ,when comparing to the basal control, at 10^{-12} M ($p<0.05$) and 10^{-10} M ($p<0.01$) (Figure 4.1A). Concentrations of 10^{-8} M 17α-oestradiol and above were stimulatory, with proliferation reaching a significant level of approximately 155% at 10^{-7} M ($p<0.05$), when comparing to the control value. The potency of the 17α- enantiomer was approximately 100-fold lower than that of 17β-oestradiol.

4.3.2 The Effect of Tamoxifen on Oestrogen-Induced ZR-75 Cell Proliferation

The specificity of the proliferative response of ZR-75 cells to 17β-oestradiol was assessed by culturing the cells with either 10^{-10} or 10^{-9} M 17β-oestradiol, previously shown to produce a sub-maximal effect (Figure 4.1A), in the presence of tamoxifen at concentrations ranging from 10^{-11} to 10^{-5} M. As illustrated in Figure 4.2, tamoxifen inhibited the response to both 10^{-9} (A) and 10^{-10} M (B) 17β-oestradiol. The effect with the higher oestrogen concentration, 10^{-9} M, was more variable (Figure 4.2A), in that the lowest (10^{-11} M) and highest (10^{-5} M) tamoxifen concentrations studied caused significant inhibition of ZR-75 cell proliferation in response to oestrogen of 50% ($p<0.01$) and 110% ($p<0.05$), respectively, whereas the inhibition achieved at concentrations between 10^{-11} and 10^{-5} M tamoxifen varied between 20 and 80% and was not significantly different to the control with oestrogen alone. By using a lower

concentration of 17β-oestradiol to induce ZR-75 cell proliferation (10^{-10} M), significant inhibition was seen with 10^{-6} M tamoxifen ($p<0.05$) (Figure 4.2B). At lower concentrations of tamoxifen a potentiation of the effect of 10^{-10} M 17β-oestradiol was seen, which was significant at 10^{-9} M tamoxifen ($p<0.001$).

4.3.3 Immunocytochemical Analysis of ER Expression by Cells and Synovial Sections

The expression of ER was studied using an immunocytochemical assay kit from Abbott Laboratories Ltd. (ER-ICA). ERs are detected by way of a specific rat anti-ER mAb, H222, which is complexed to a bridging anti-rat IgG antibody and hence to a peroxidase/anti-peroxidase complex. Finally a chromogen-substrate solution containing hydrogen peroxide and diaminobenzidine 4 HCl is added to allow visualisation of the receptors. Synovial fibroblasts, PBMC, ZR-75 and Hs578T cells and sections of RA synovial tissue were prepared and examined using the kit. The control slides supplied for use with the ER-ICA kit gave positive reddish-brown nuclear staining of varying intensities with the H222 antibody (Figure 4.3A (+)), compared to negative staining obtained from incubation with the control antibody (normal rat antibody) (Figure 4.3A (-)). Positive staining was also seen with the H222 mAb using preparations of ZR-75 cells which had been maintained in oestrogen-supplemented medium (Figure 4.3B (+)), although slightly less intense than that obtained with cells on the control slide, whereas ZR-75 cells which were cultured in oestrogen-depleted medium for 7 days failed to demonstrate ERs (Figure 4.3C (+)). The relevant negative staining for ZR-75 cells, with control antibody, is shown in Figures 4.3B and C (-). Hs578T were essentially negative, as shown in Figure 4.3D, and incubation of PBMC from controls or RA patients with the H222 mAb also failed to detect expression of ER. An example of the negative results obtained are illustrated with PBMC from a female RA patient (Figure 4.3E). In addition, staining of synovial fibroblasts and RA synovium with the ER-ICA kit gave negative results (Figures 4.3F and G, respectively). Attempts to visualise ER using the H222 mAb by FACS analysis were unsuccessful with all cell types and studies using a different anti-ER mAb resulted in a high level of non-specific staining, hence this latter antibody could not be used in further experiments (results not shown).

4.3.4 Preparation of Cells for FACS Analysis of ER Expression

Examination of ER expression by FACS analysis involves permeabilising the cell membrane prior to incubating with the anti-ER mAb, as the receptor occurs intracellularly. The saponin-permeabilisation technique did not significantly alter cell morphology, as illustrated by the dot plots of granularity (side scatter, SSC) versus size (forward scatter, FSC) in Figure 4.4 for ZR-75 cells (A), Hs578T cells (B), synovial fibroblasts (C) and PBMC (D). To ensure that cells were permeabilised using this method, PBMC were incubated with antibodies directed against two intracellular proteins, namely centromere and topoisomerase. Figure 4.5B demonstrates that the cells were indeed permeabilised as a result of saponin treatment as there was a significant increase in fluorescence with the two antibodies compared to that with the control antibody, whereas neither of the antibodies bound to non-permeabilised cells (Figure 4.5A). Cells were routinely fixed with 1% (w/v) paraformaldehyde following preparation for FACS analysis, prior to analysis. Figure 4.6 illustrates that PBMC morphology, in terms of size and granularity, was not altered by this fixation method. This applied to all cell types studied (results not shown).

Prior to the analysis of fluorescence changes as a result of labelling with specific mAbs, cells which had been incubated with control antibody were observed, and a homogeneous cell population selected. This was achieved by gating the area of greatest fluorescence intensity, as shown in Figure 4.7 for ZR-75 cells (A), Hs578T cells (B), synovial fibroblasts (C) and PBMC (D). With PBMC preparations, the aim was to select the T cell/monocyte population and exclude any contaminating cell types, such as PMNL. The purity of the gated cells was assessed by staining with an anti-CD3 mAb. This demonstrated that the majority of the PBMC included within the gate were T cells and hence stained positively with the anti-CD3 mAb (Figure 4.7D). Very few T cells were excluded, and the non-T cell population included within the gated region was a minority and assumed to be monocytes.

4.3.5 The Expression of p29 Antigen by Cells and Synovial Sections

The failure of the H222 mAb to detect ER in all but the ZR-75 cell line led to the use of the ER-D5 mAb, which recognises a 29 kD human-specific cytoplasmic, non-hormone-binding protein associated with the ER. Cells were examined by FACS analysis following the saponin-permeabilisation method of preparing cells, and all cell types studied were gated as described in Section 4.3.4. Figure 4.8 illustrates histograms of

fluorescence intensity. Saponin-permeabilised ZR-75 cells (A), Hs578T cells (B), synovial fibroblasts (C) and PBMC (D) all stained positively with the ER-D5 mAb (second peak), shown as a shift in fluorescence compared to that with the control antibody (first peak). All results depict the change in fluorescence with a 1/10 dilution of ER-D5, therefore a comparison can be made in terms of cellular expression of the p29 antigen. In this respect, the order of expression of the p29 antigen was seen to be: ZR-75 cells > synovial fibroblasts > Hs578T cells > PBMC. In contrast there was no change in the basal level of fluorescence with ER-D5 mAb when cells used were non-permeabilised. Therefore, the expression of the p29 antigen is exclusively intracellular.

The results obtained for p29 expression by FACS analysis were comparable to those obtained by immunocytochemistry. Cells and RA synovial tissue were prepared and stained with the ER-D5 mAb using the APAAP technique. Intense cytoplasmic staining was seen with ZR-75 cells (Figure 4.10A (+)) and synovial fibroblasts (Figure 4.10E (+)), with the expression of p29 greater in the former cell type. All cells tested with the ER-D5 mAb were also incubated with a control antibody, which consistently produced negative results (Figure 4.10 (-)). PBMC from both controls and RA patients incubated with ER-D5 showed weak staining which appeared to be more diffuse than with ZR-75 cells or synovial fibroblasts. The staining was more intense for the female RA patient (Figure 4.10D (+)) than for the female control (Figure 4.10C (+)) illustrated, in this instance, although in general no trend was seen for male and female controls and RA patients of various ages using the APAAP procedure. When synovial tissue sections from a postmenopausal female RA patient were tested with the ER-D5 mAb, specific areas of positive and negative staining were seen (Figure 4.10F (+)). There was weak, diffuse staining of the synovial lining layer and endothelial cells appeared to be negative for p29 expression. In contrast, there were intense areas of staining which occurred in distinct clusters of infiltrating cells and which seemed to be cell-specific. This was observed in sequential sections and thus was unlikely to be artefactual (results not shown).

4.3.6 FACS Analysis of p29 Antigen Expression by Control and RA PBMC

A comparison was made between p29 expression in PBMC from RA patients and age- and sex-matched normal controls. Cells were prepared for FACS analysis using ER-D5 mAb at dilutions ranging from 1/6.25 to 1/100 and the results obtained as either % positive cells or MFI. In contrast to the results illustrated in Figure 4.10C and D

showing APAAP staining of control and RA PBMC with the ER-D5 mAb, the expression of p29 antigen in RA PBMC was seen to be considerably less than that with control PBMC, at all mAb dilutions, in terms of the number of cells expressing the antigen (% positive cells) (Figure 4.9A) or the number of antigenic sites per cell (MFI) (Figure 4.9B). However, when comparing control and RA data using a Mann-Whitney U Test, only the 1/100 dilution of ER-D5, when considering MFI, showed a significant difference ($p<0.05$) (Figure 4.9B).

4.3.7 Analysis of ER mRNA Expression by Northern Blotting and RT-PCR

As attempts to localise ER protein in PBMC and synovial fibroblasts were unsuccessful, except for the demonstration of the ER-related antigen p29, experiments were carried out to investigate ER mRNA expression. RNA was prepared from ZR-75, T-47D and Hs578T cells and control PBMC and Northern blotted. The filter was hybridised to an ER cDNA probe which initially failed to detect any mRNA species, although a Southern blot constructed with ER plasmid DNA and hybridised to the ER cDNA demonstrated that the probe was capable of hybridising to itself (Figure 4.11). A GAPDH probe was used to ensure that the RNA had not been removed from the surface of the Northern blot filter. Figure 4.12A demonstrates that the filter was able to hybridise to the GAPDH cDNA and that the RNA was therefore intact. Hence, various hybridisation techniques were tested with the Northern blot filter, and one procedure was successful in detecting a very faint band which corresponded to the 6.2 kb transcript for ER mRNA (see Method 4.2.3) (Figure 4.12B). The band was seen for ZR-75 cells only. Further purification of total RNA to poly(A) RNA (ie. mRNA enriched) did not improve the ability of the ER cDNA to hybridise (results not shown).

RT-PCR has been developed as an extremely sensitive method for detecting gene expression at the RNA level. Oligonucleotide primers designed to frame a 287 bp segment of human cDNA within the oestrogen-binding domain (see Method 4.2.4) were initially tested using human ER-containing plasmid DNA, which resulted in a PCR product of the expected size (results not shown). Therefore, RNA from ZR-75, T-47D and Hs578T cells, synovial fibroblasts and from both control and RA PBMC was prepared and reverse transcribed to single stranded cDNA which was then amplified by the PCR, using this set of oligonucleotide primers. As shown in Figure 4.14A the expected 287 bp fragment was amplified from both the ZR-75 and T-47D cell lines (lanes 1-3 and lane 6, respectively), whereas no product was amplified for the

Hs578T cell line (lane 4). A PCR reaction with no RNA or DNA added was also negative (results not shown). PBMC RNA reverse transcribed and amplified to give the 287 bp fragment and a slower migrating species was also detected (lane 5). ZR-75 cells demonstrated the greatest amount of product. Synovial fibroblast RNA also showed a 287 bp fragment on amplification (Figure 4.14B). RNA from a range of premenopausal and male controls was analysed with the results demonstrated in Figure 4.15A and B, with the amount of the 287 bp product obtained differing between donors. PBMC RNA from one of the female controls gave two bands (Figure 4.15A, lane 5) and with two of the male control PBMC samples the 287 bp fragment was either undetectable (Figure 4.15B, lane 4), or present at only a very low level (Figure 4.15B, lane 3). RA PBMC also demonstrated the RT-PCR 287 bp product (Figure 4.15C), although bands were too faint to determine whether there were any quantitative differences in expression between the peri- and postmenopausal female and male patients examined.

The unavailability of NBL Taq polymerase enzyme after initial RT-PCR experiments resulted in the use of Promega and then Perkin-Elmer Taq polymerase enzymes. The NBL enzyme was supplied with a $MgCl_2$-containing buffer, whereas the latter were supplied with $MgCl_2$-free buffers. Hence $MgCl_2$ titrations were carried out, with concentrations ranging from 0.5 to 6.0 mM. As demonstrated in figure 4.13A and B, a concentration of 1.5 mM $MgCl_2$ gave optimal amplification of ZR-75 cDNA, and thus this was used for all PCR reactions with these two enzymes. Interestingly, concentrations of Perkin-Elmer Taq polymerase enzyme above 1.5 mM also resulted in a second slower-migrating species (Figure 4.13B). Initial experiments used M-MLV reverse transcriptase, but as the RNase H- Superscript reverse transcriptase enzyme was more efficient in terms of the amount of final product achieved after PCR (Figure 4.13C), this was used for later experiments.

The 287 bp fragment amplified from ZR-75 cells was sequenced and found to be identical to the corresponding region of the human cDNA (Greene *et al.*, 1986) (results not shown). An attempt was made to separate, and hence sequence, the two products seen with certain PBMC samples using a band-stab PCR method (see Method 4.2.6). However, this consistently resulted in one PCR product. Alternative methods of purification were also unsuccessful and therefore the identity of the unknown PCR product could not be assessed. Further analysis of ER mRNA expression using the RT-PCR method was hindered by experimental difficulties, and attempts to quantify the PCR and hence make direct comparisons between the level of ER mRNA expression in different cell types were also unsuccessful (results not shown).

4.4 SUMMARY

Results demonstrated that the ZR-75 cell line responded to 17β-oestradiol in a dose-dependent manner, with an increase in proliferation. The 17α-enantiomer also stimulated ZR-75 cell proliferation, but was approximately 100-fold less active. Neither the T-47D or Hs578T cell lines proliferated in response to 17α- or 17β-oestradiol. The proliferative response of ZR-75 cells in response to 17β-oestradiol was inhibited with the anti-oestrogen tamoxifen. ER was detected in ZR-75 cells at the protein and mRNA level, whereas with T-47D cells, ER protein could not be detected although the mRNA was present. Hs578T cells were ER-negative. ER mRNA was detected in both control and RA PBMC and in synovial fibroblasts, but the protein was undetectable with the assay systems employed. Using a mAb directed against an ER-related protein, p29, all cells tested demonstrated positivity to a certain extent. When comparing PBMC from normal controls and RA patients, the rheumatoid cells appeared to express less p29, although this was not significant.

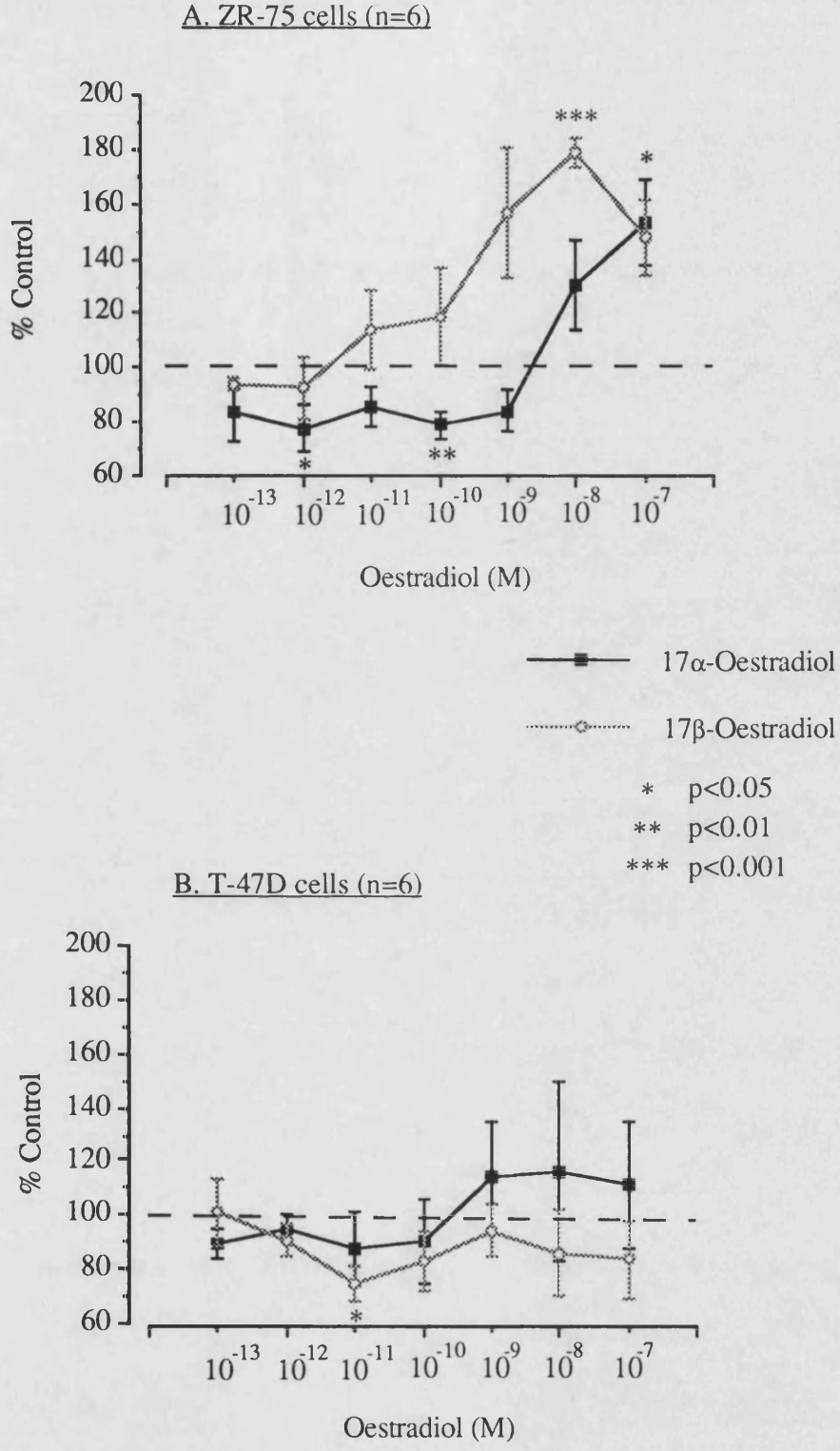


Figure 4.1 Continued...

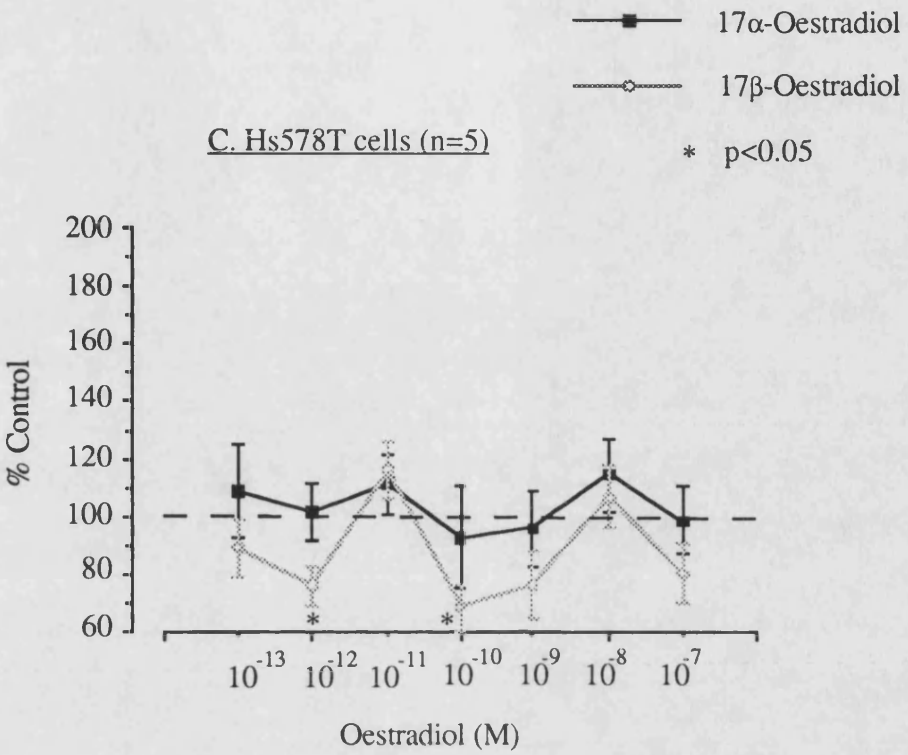


Figure 4.1 The effect of oestrogen on ZR-75, Hs578T and T-47D cell proliferation

ZR-75, Hs578T and T-47D cells were cultured with 10^{-13}-10^{-7} M 17α- or 17β-oestradiol. After 72 hours incubation cells were pulsed with [^{3}H]thymidine, harvested, counted and proliferation assessed. Tests was performed in triplicate and the three values (CPM) combined to give an average. The control, ie. 72 hour proliferation in the absence of hormone, was set at 100% and results are expressed relative to this. Each point represents the mean ± SEM of five or six separate experiments. Data was analysed using a paired Student's T-test by comparing to the control value.

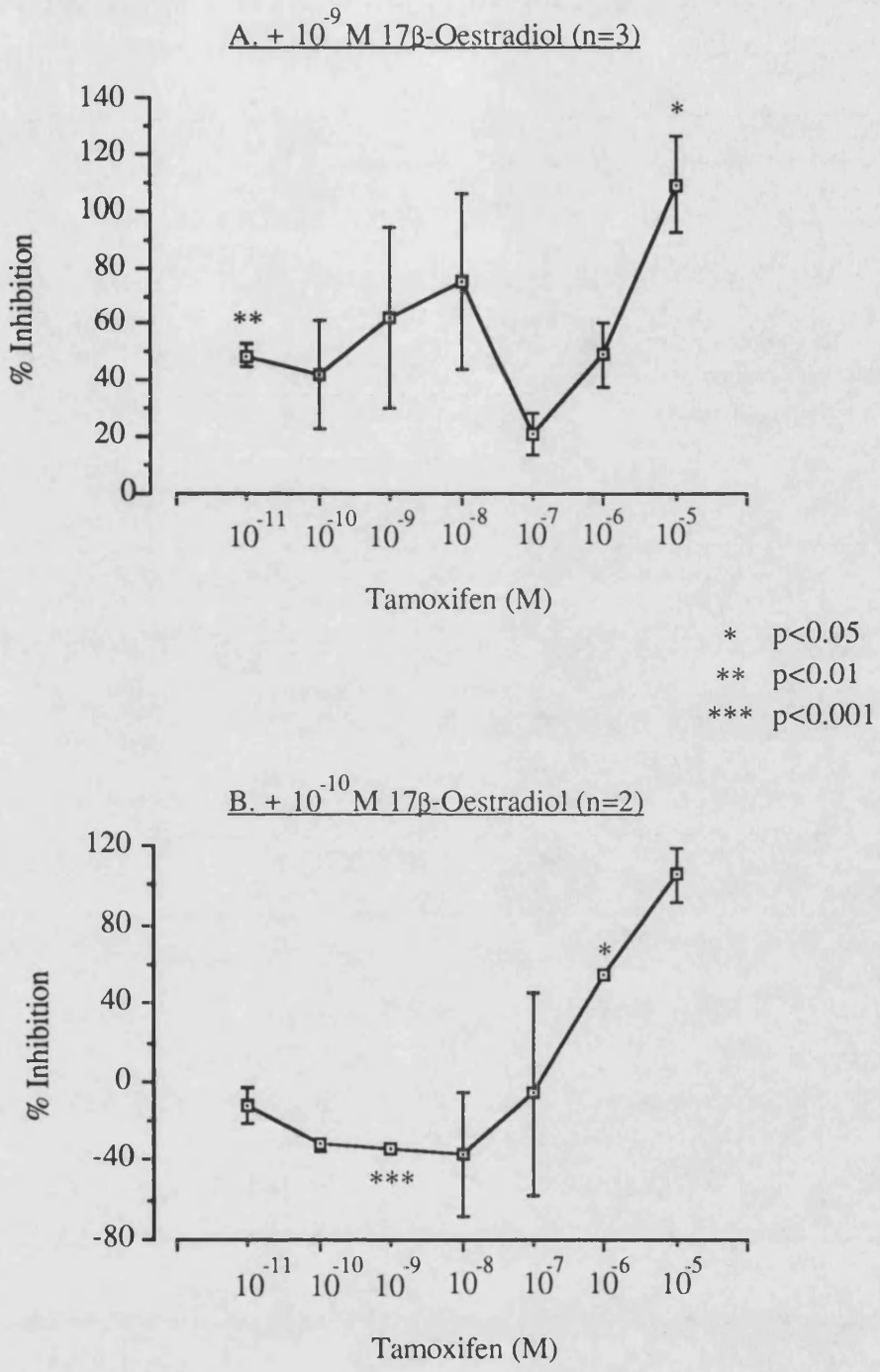
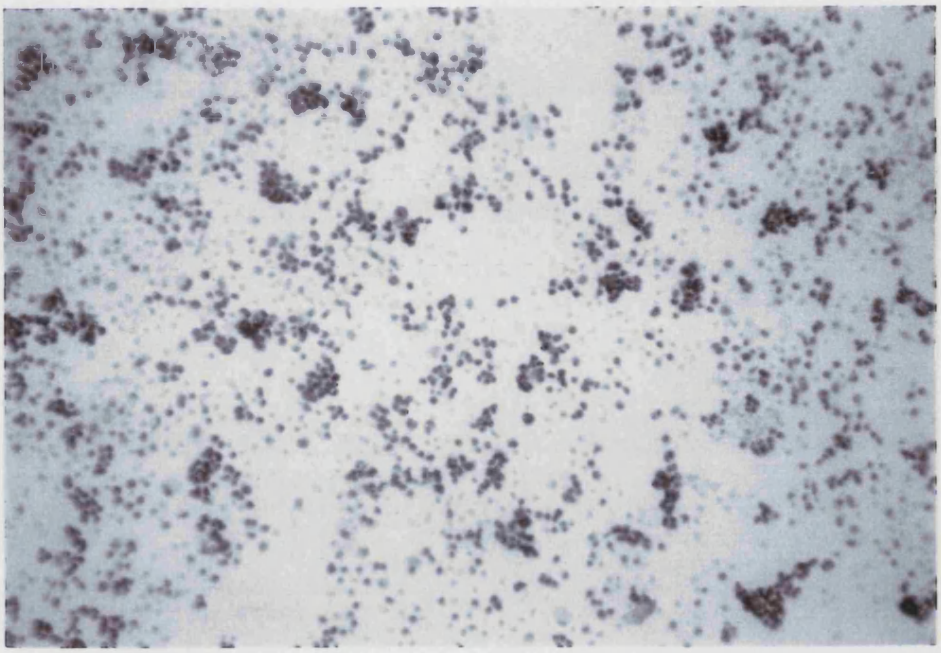


Figure 4.2 The effect of tamoxifen on oestrogen-induced ZR-75 cell proliferation

ZR-75 cells were cultured with (A) 10^{-9} M 17β-oestradiol or (B) 10^{-10} M 17β-oestradiol in the presence or absence of 10^{-11}-10^{-5} M tamoxifen. After 72 hours incubation cells were pulsed with [^{3}H]thymidine, harvested, counted and proliferation assessed. Tests were performed in triplicate and the three values (CPM) combined to give an average. The negative control, ie. cells cultured in the absence of 17β-oestradiol and tamoxifen, was set at 100%, and the positive control, ie. cells cultured in the presence of 17β-oestradiol alone, was set at 0%. All data was then expressed as % inhibition relative to this. Each point represents the mean ± SEM of two or three separate experiments. Data was analysed using a paired Student's T-test by comparing to the positive control value.

A. Control slide

(+)



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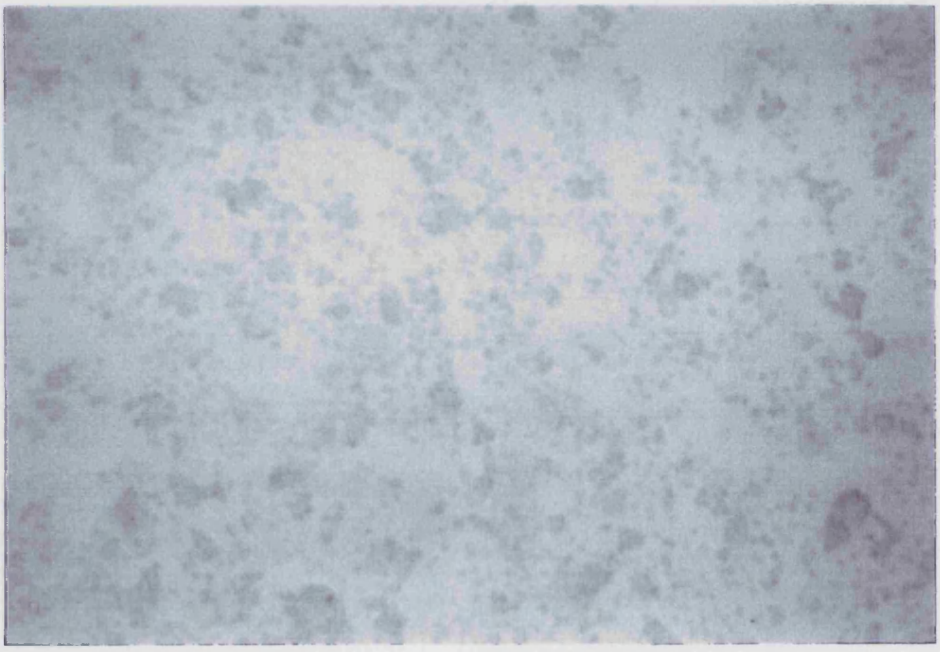
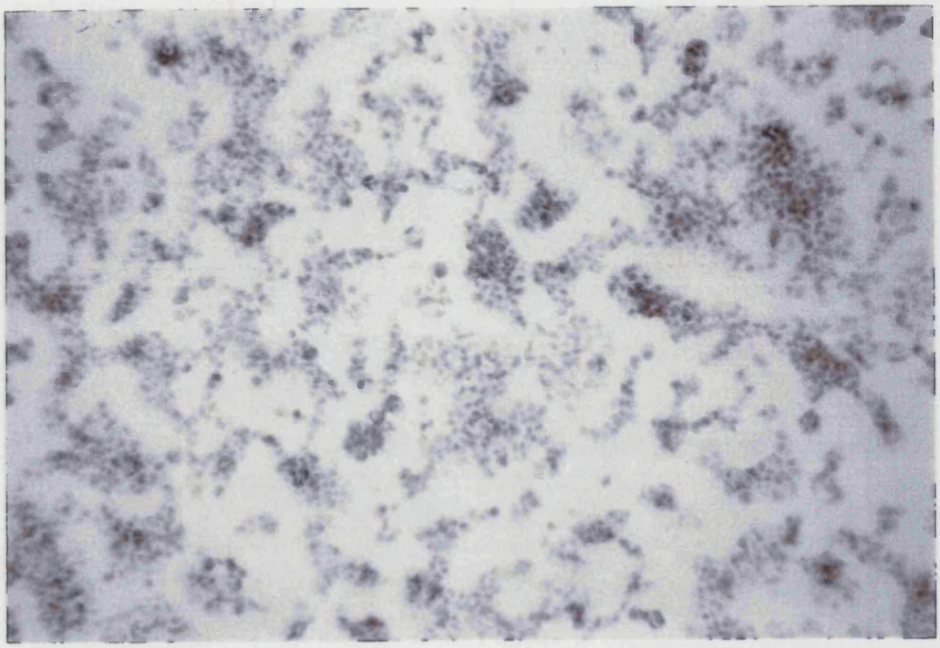


Figure 4.3 Continued...

B. ZR-75 cells (maintained in oestrogen-supplemented medium)

(+)



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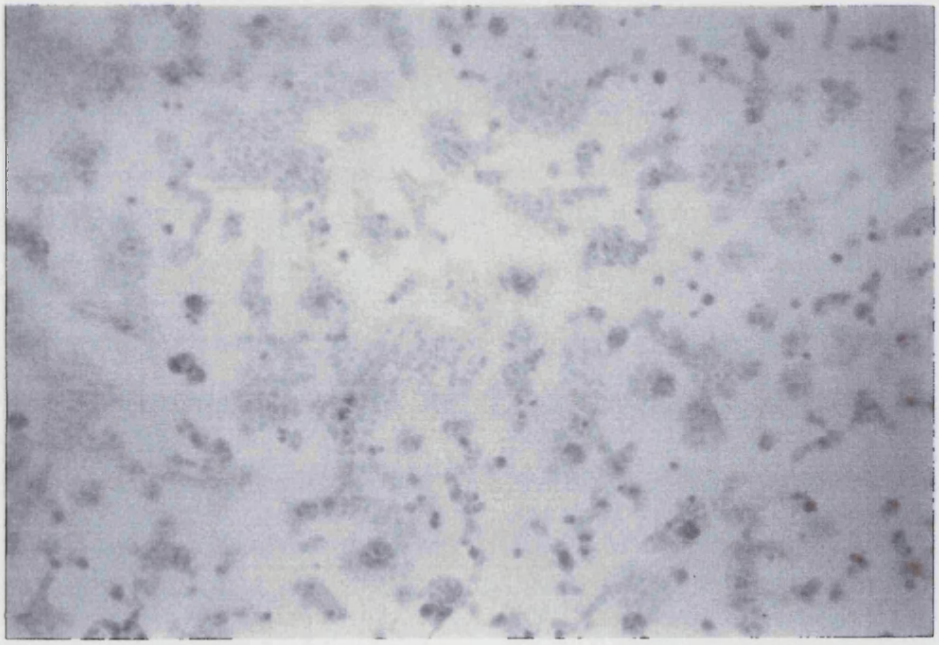
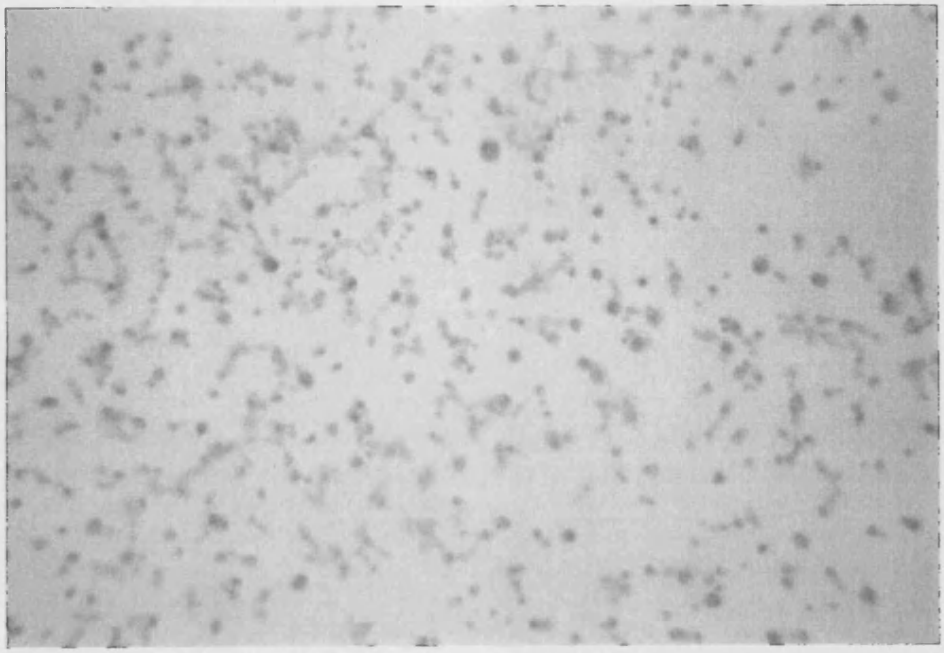


Figure 4.3 Continued...

C. ZR-75 cells (maintained in oestrogen-depleted medium)

(+)



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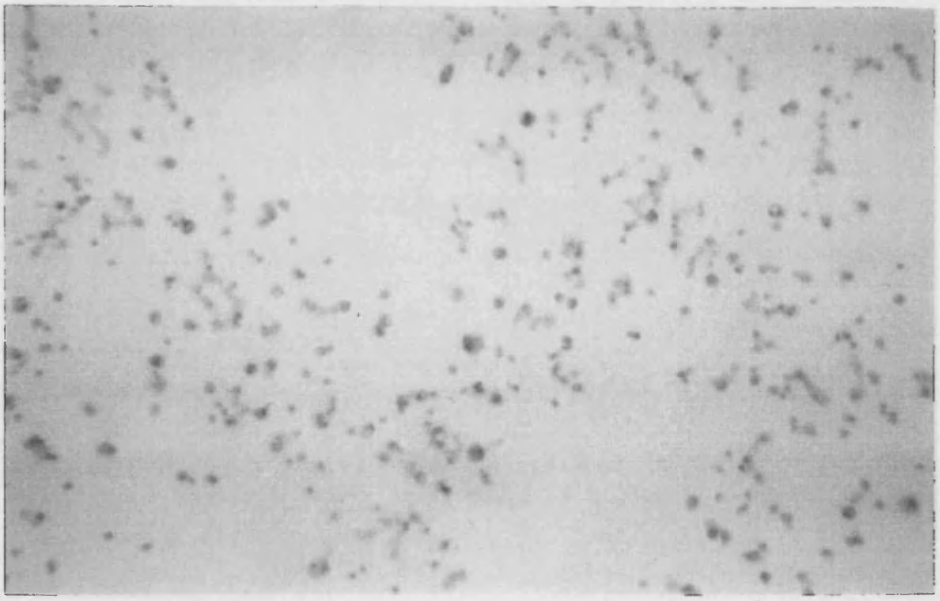

Figure 4.3 Continued...

D. Hs578T cells

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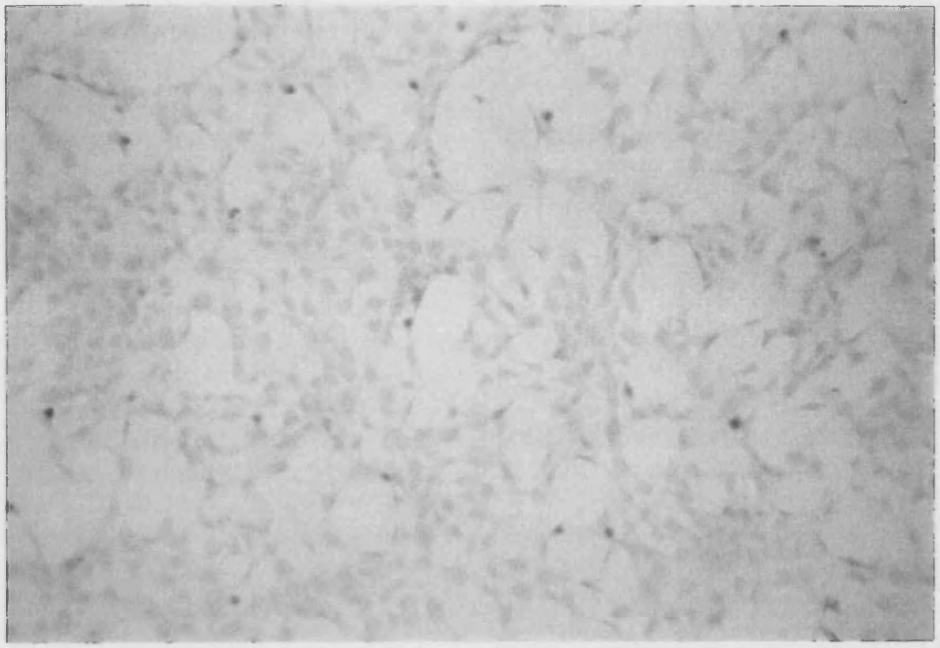


E. PBMC

(+)

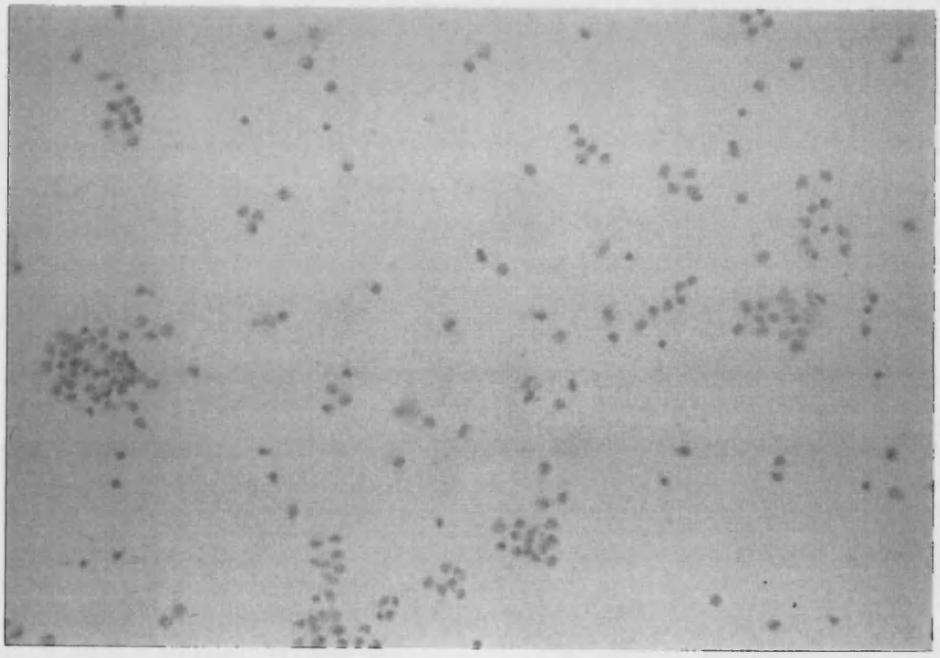
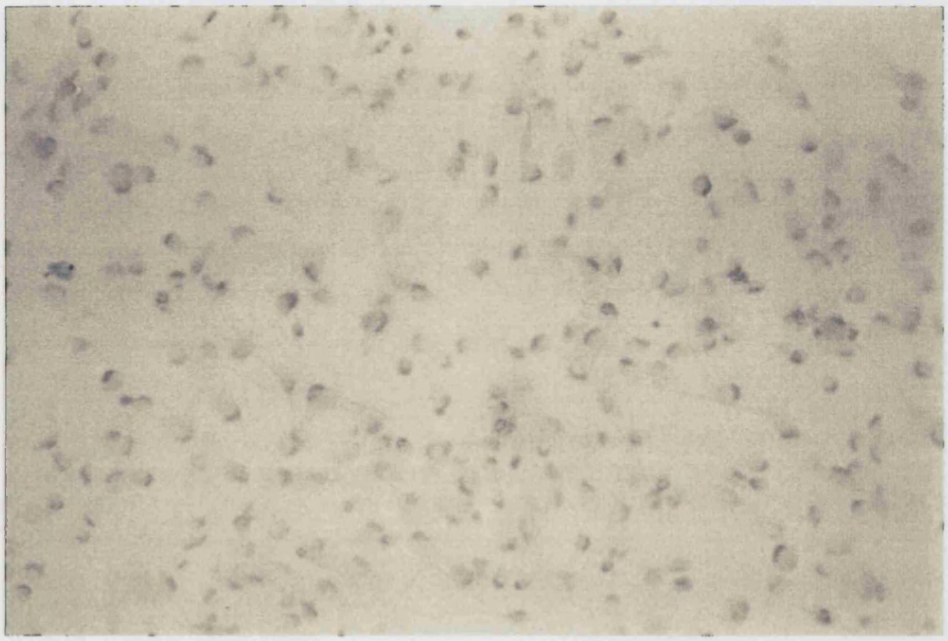


Figure 4.3 Continued...

F. Synovial fibroblasts

(+)



G. RA synovial tissue

(+)

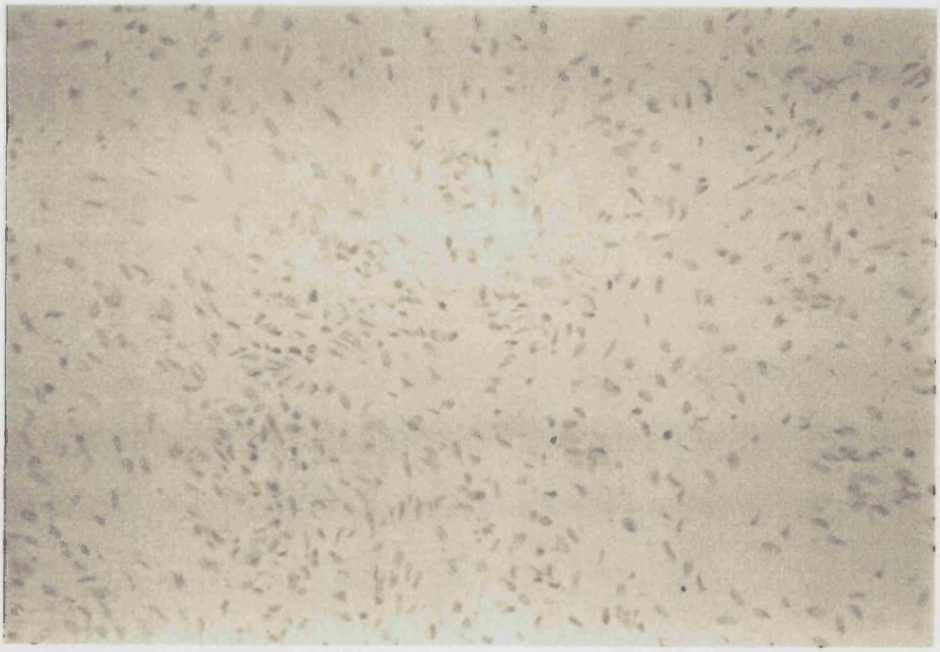
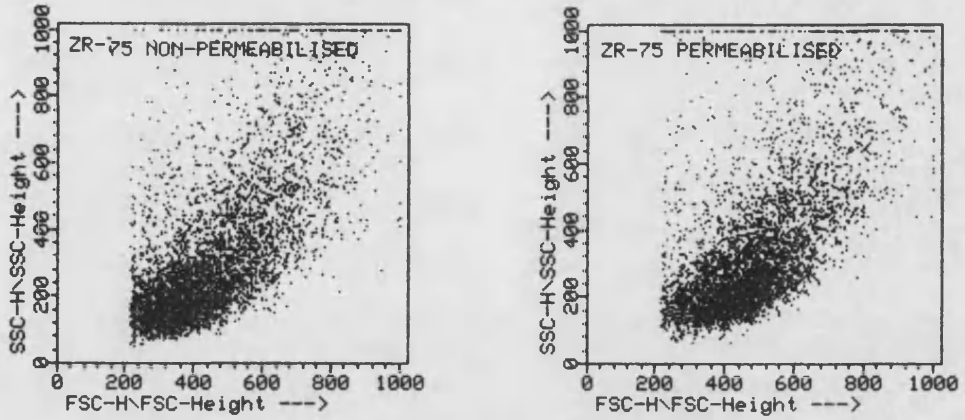


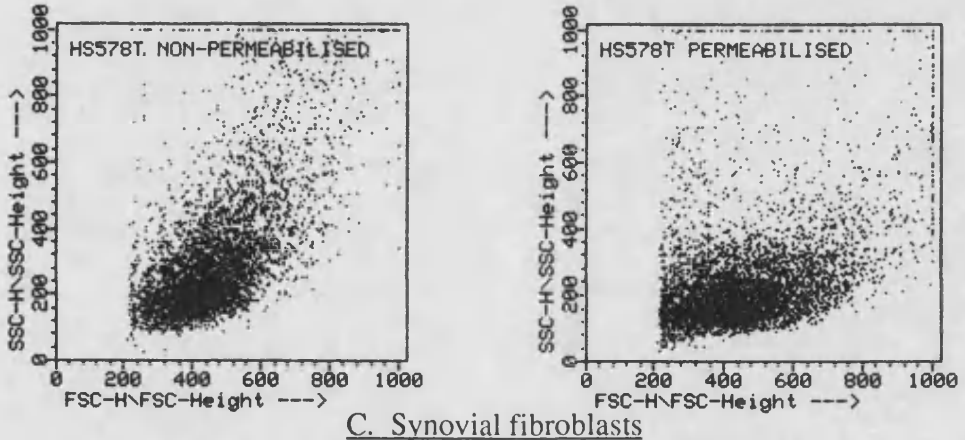
Figure 4.3 Detection of oestrogen receptor in cells and synovial sections

ZR-75 cells maintained in oestrogen-supplemented medium (B) or oestrogen-depleted for 7 days (C), Hs578T cells (D), PBMC (E), synovial fibroblasts (F) and RA synovial tissue (G) were stained with an anti-ER mAb (H222) (+) or a control antibody (-), using an Abbott ER-ICA kit. Results were compared with those obtained with control slides (A). (Original magnification x66 (A-F) and x33 (G)).

A. ZR-75 cells



B. Hs578T cells



C. Synovial fibroblasts

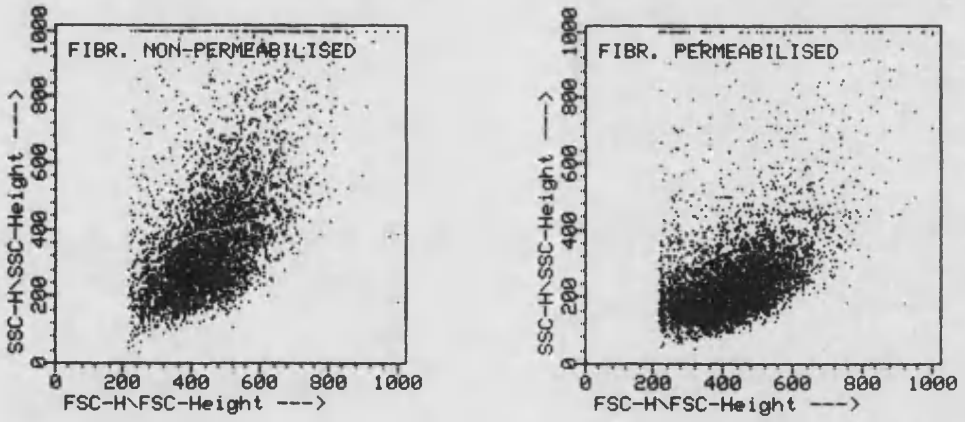


Figure 4.4 Continued...

D. PBMC

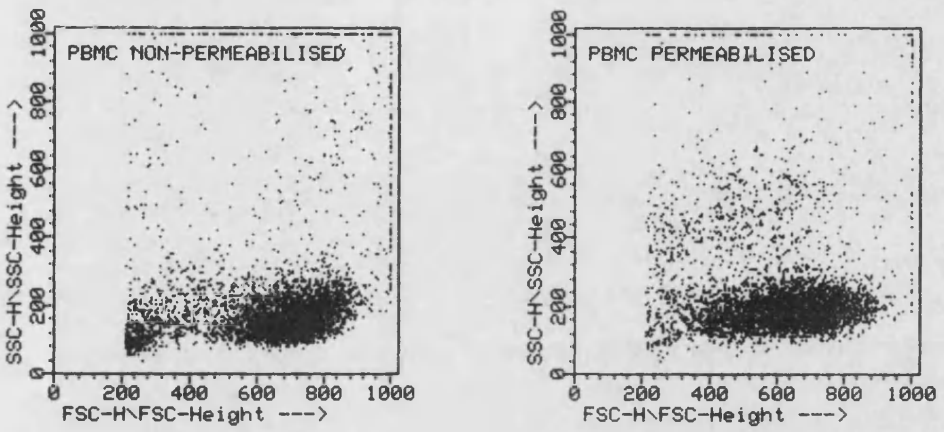
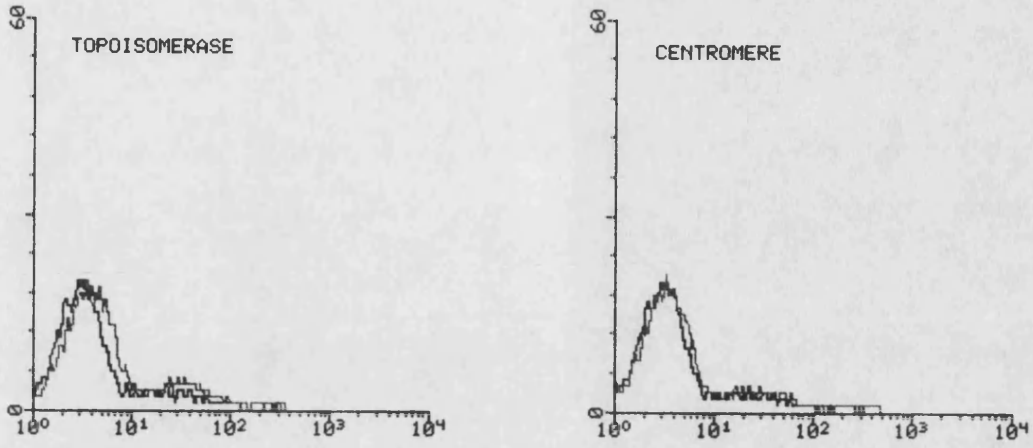


Figure 4.4 The effect of saponin permeabilisation on cellular integrity

(A) ZR-75 cells, (B) Hs578T cells, (C) synovial fibroblasts and (D) PBMC were either non-permeabilised or were saponin-permeabilised, prior to being incubated with mouse IgG followed by an anti-mouse IgG FITC-conjugate, and cellular integrity determined by FACS analysis. Results are expressed as dot-plots of side scatter (SSC) versus forward scatter (FSC).

A. Non-permeabilised PBMC



B. Saponin-permeabilised PBMC

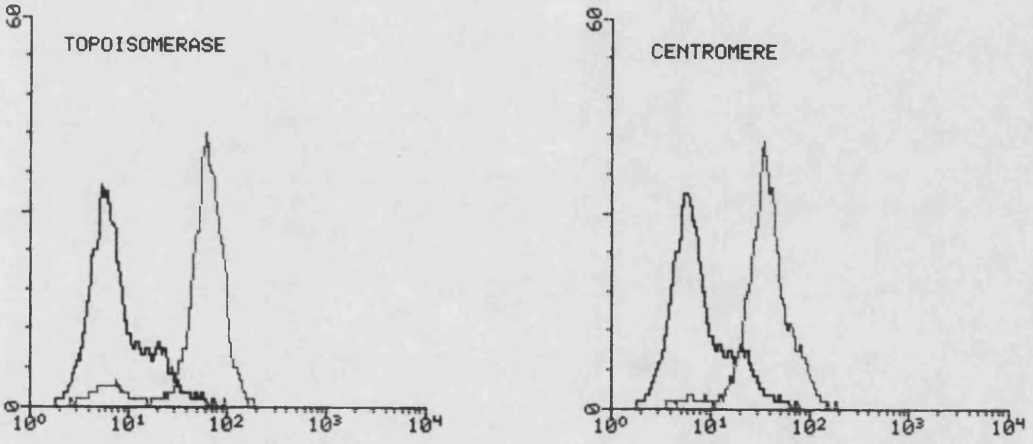


Figure 4.5 Assessing the effectiveness of saponin permeabilisation

PBMC were either (A) non-permeabilised or (B) saponin-permeabilised prior to being incubated with anti-topoisomerase or anti-centromere antibodies at 1/100 dilution, or the equivalent concentration of human IgG, followed by an anti-human IgG FITC-conjugate. Cells were then examined by FACS analysis. Results are expressed as histograms of fluorescence intensity, with the first peak representing the control IgG and the second peak representing the test antibody.

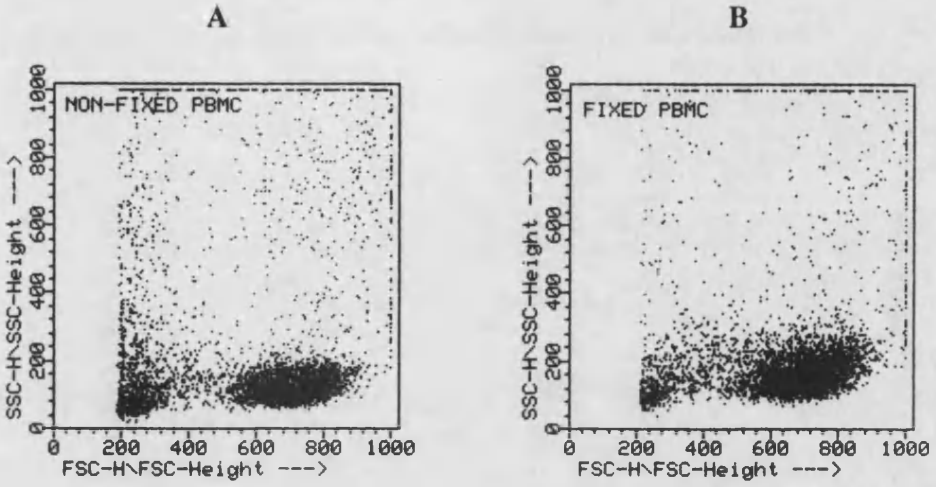
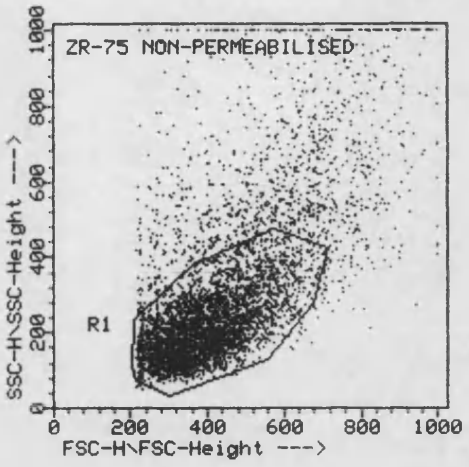


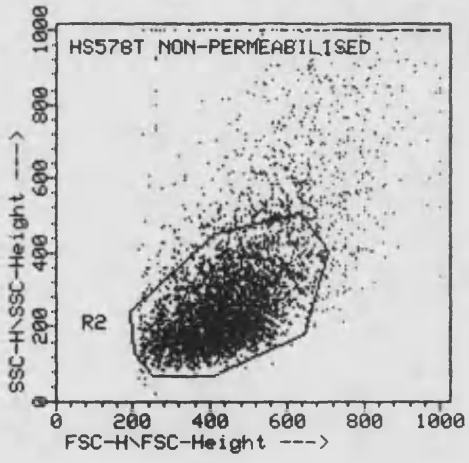
Figure 4.6 The effect of paraformaldehyde fixation on cellular integrity

PBMC were incubated with mouse IgG followed by an anti-mouse IgG FITC-conjugate and were either examined by FACS analysis immediately (A), or were fixed with 1% (w/v) paraformaldehyde prior to analysis (B). Results are expressed as dot-plots of side scatter (SSC) versus forward scatter (FSC).

A. ZR-75 cells



B. Hs578T cells



C. Synovial fibroblasts

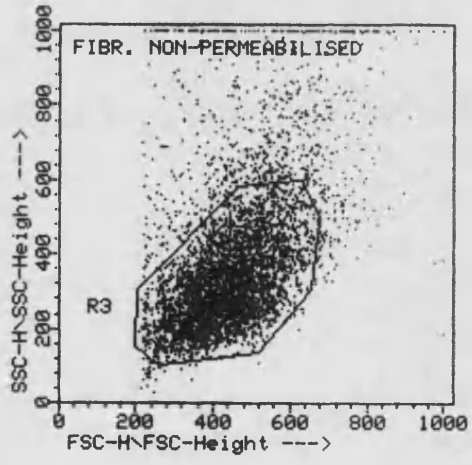


Figure 4.7 Continued...

D. PBMC

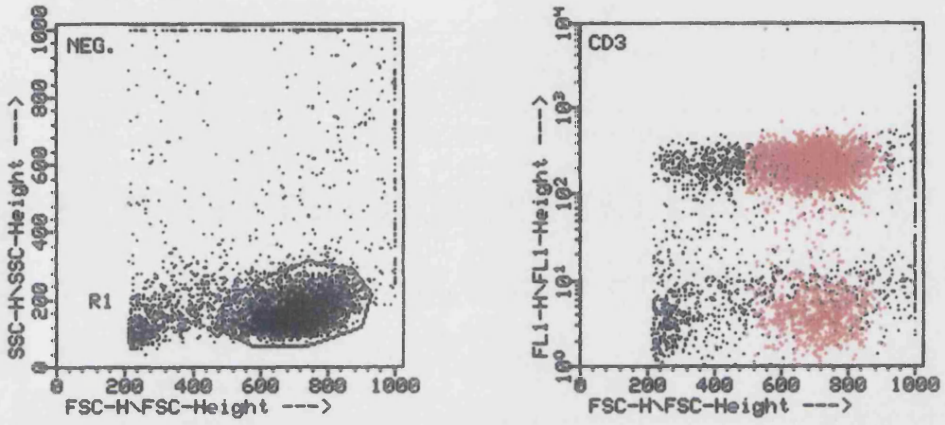
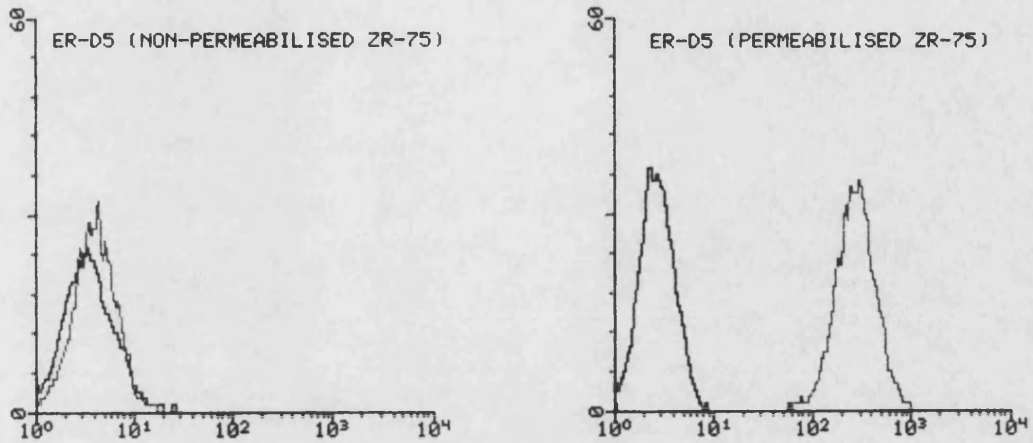


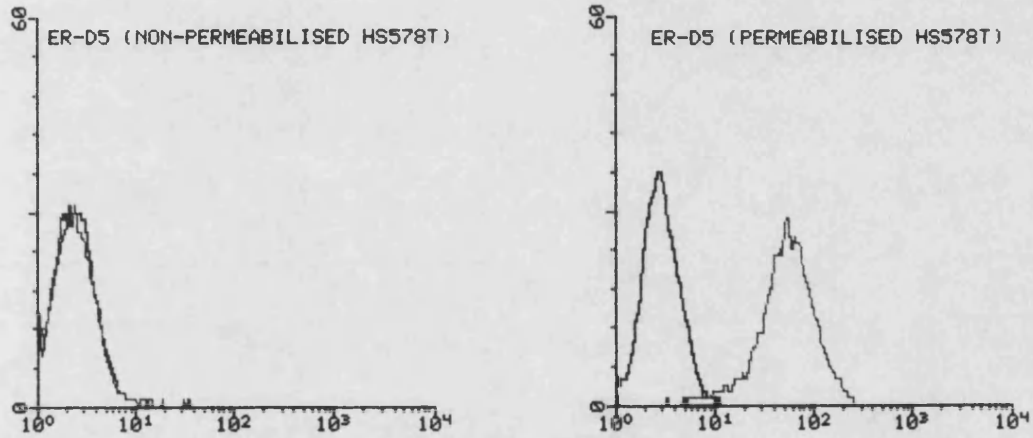
Figure 4.7 Obtaining a homogeneous cell population for FACS analysis

Cells were prepared for FACS analysis and, prior to observing fluorescence intensities with the mAbs under examination, a homogeneous cell population was selected. This was achieved by observing a dot-plot of side scatter (SSC) versus forward scatter (FSC) and setting a gate which encompassed the area of greatest cellular intensity. Results demonstrate typical homogeneous cell populations selected for (A) ZR-75 cells, (B) Hs578T cells, (C) synovial fibroblasts. PBMC were compared after incubating with control antibody (Neg.) or anti CD3 mAb (CD3), with the gate set for the control antibody and the percentage of anti-CD3 positive cells occurring within the gate observed (D).

A. ZR-75 cells



B. Hs578T cells



C. Synovial fibroblasts

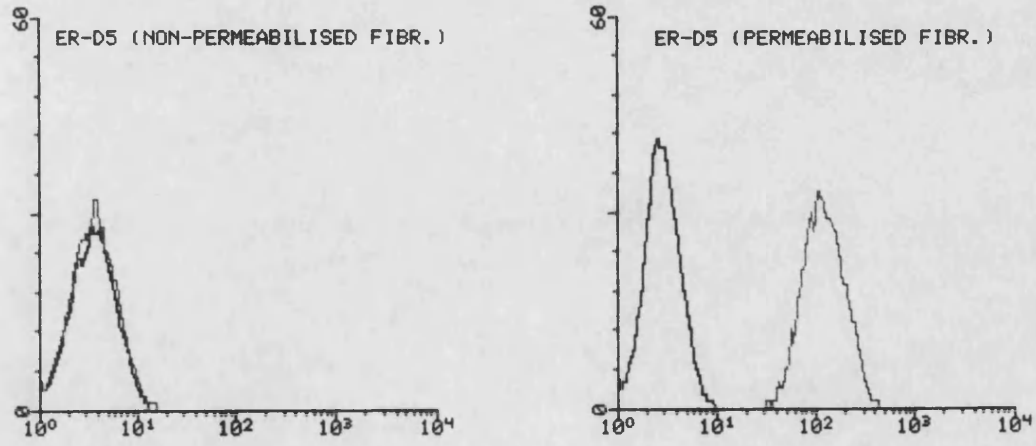


Figure 4.8 Continued...

D. PBMC

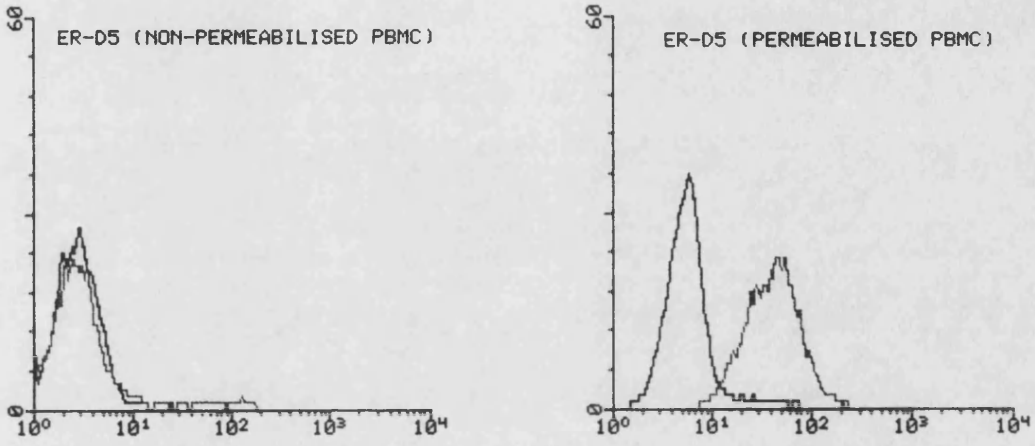


Figure 4.8 FACS analysis of the cellular expression of p29 antigen

(A) ZR-75 cells, (B) Hs578T cells, (C) synovial fibroblasts and (D) PBMC were either untreated or saponin-permeabilised prior to being incubated with ER-D5 mAb at 1/10 dilution, or the equivalent concentration of mouse IgG, followed by an anti-mouse IgG FITC-conjugate. Cells were then examined by FACS analysis. Results are expressed as histograms of fluorescence intensity, with the first peak representing the control IgG and the second peak representing the mAb.

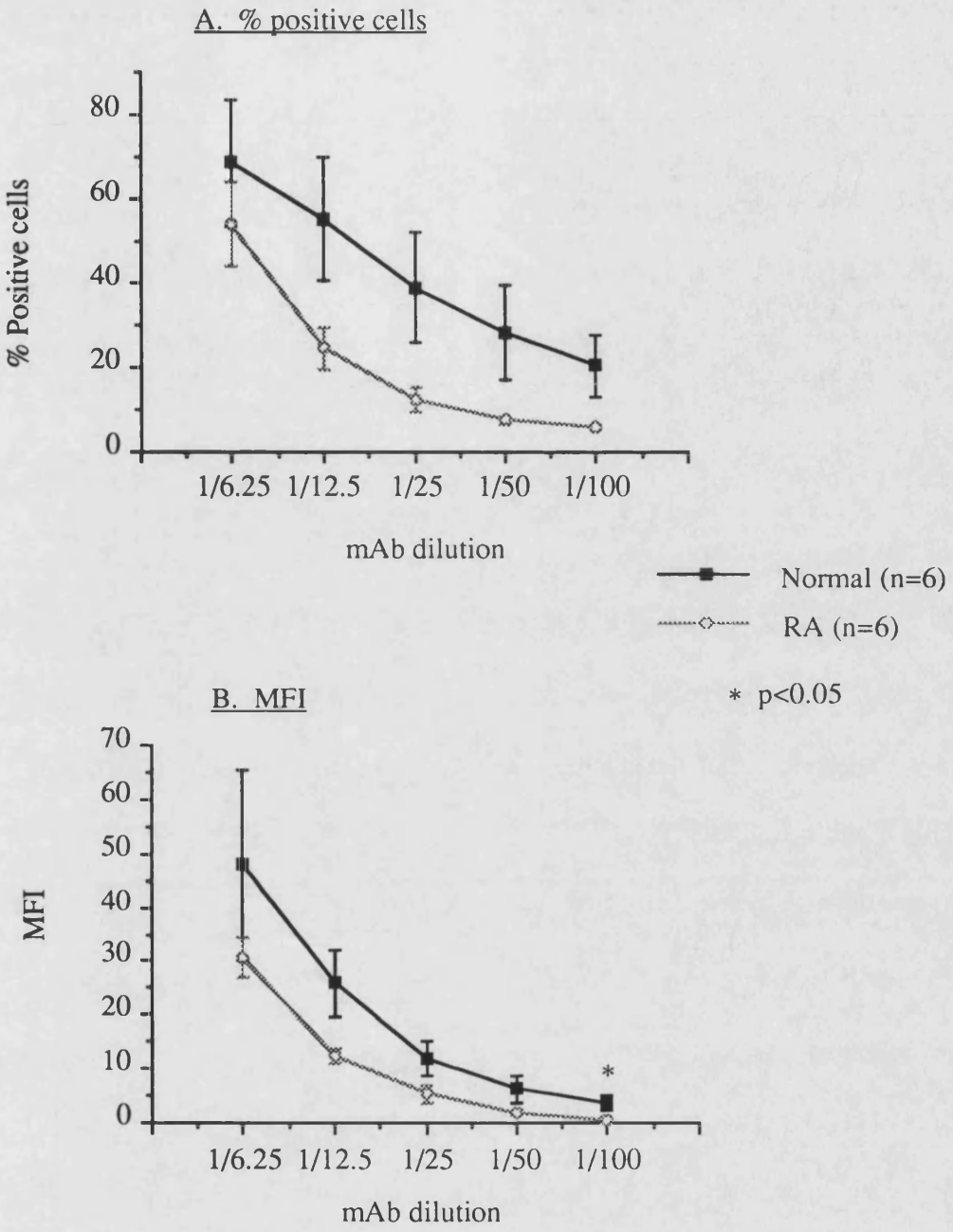
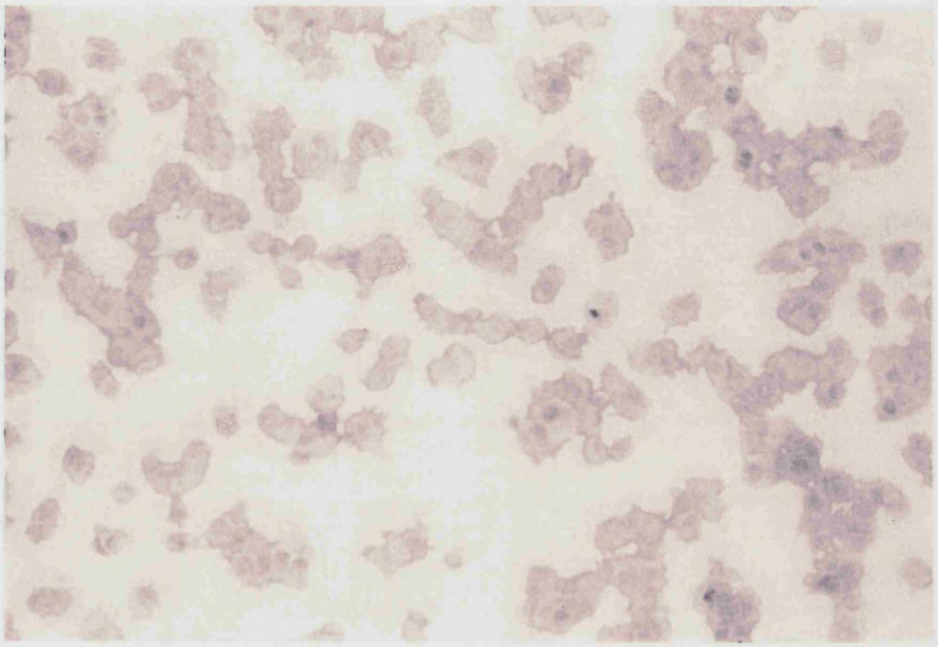


Figure 4.9 FACS analysis of p29 antigen expression in control and RA PBMC

PBMC were obtained from pre-, peri- and postmenopausal female and male RA patients and age-and sex-matched healthy controls. Cells were saponin-permeabilised and incubated with ER-D5 mAb at varying concentrations, or mouse IgG at a concentration equivalent to the highest mAb dilution, followed by an anti-mouse IgG FITC-conjugate. Cells were then examined by FACS analysis. Each dilution was performed in duplicate and the two values obtained were combined to give an average. Results are expressed as (A) % positive cells, relative to the control IgG which is set at 5%, or (B) mean fluorescence intensity (MFI), which has been corrected for the control IgG. Each point is the mean ± SEM of six separate experiments. Data was analysed using the Mann-Whitney U test by comparing values for the RA group to those for the controls.

A. ZR-75 cells

(+)



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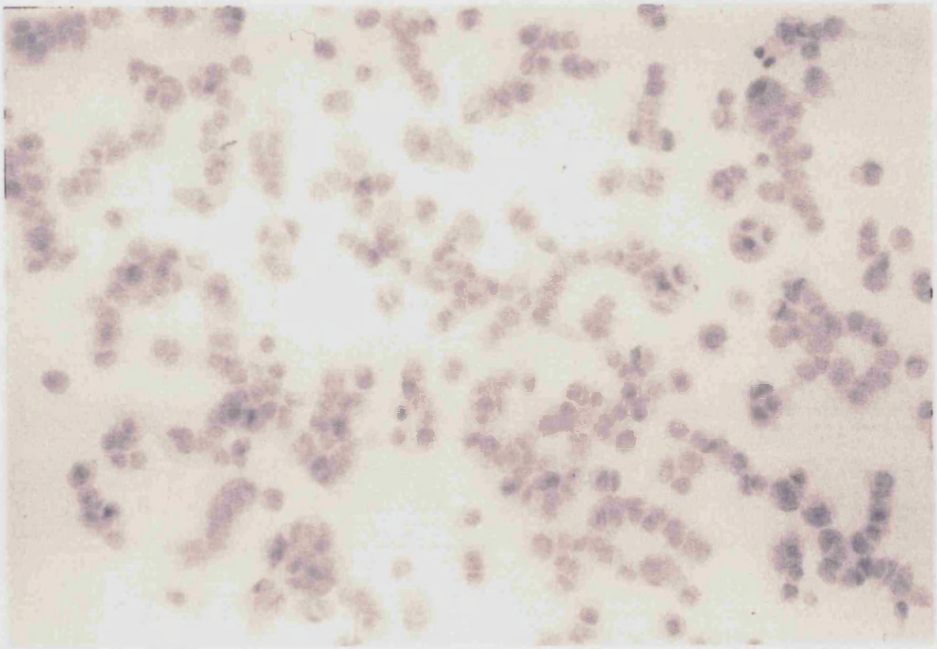

Figure 4.10 Continued...

B. Hs578T cells

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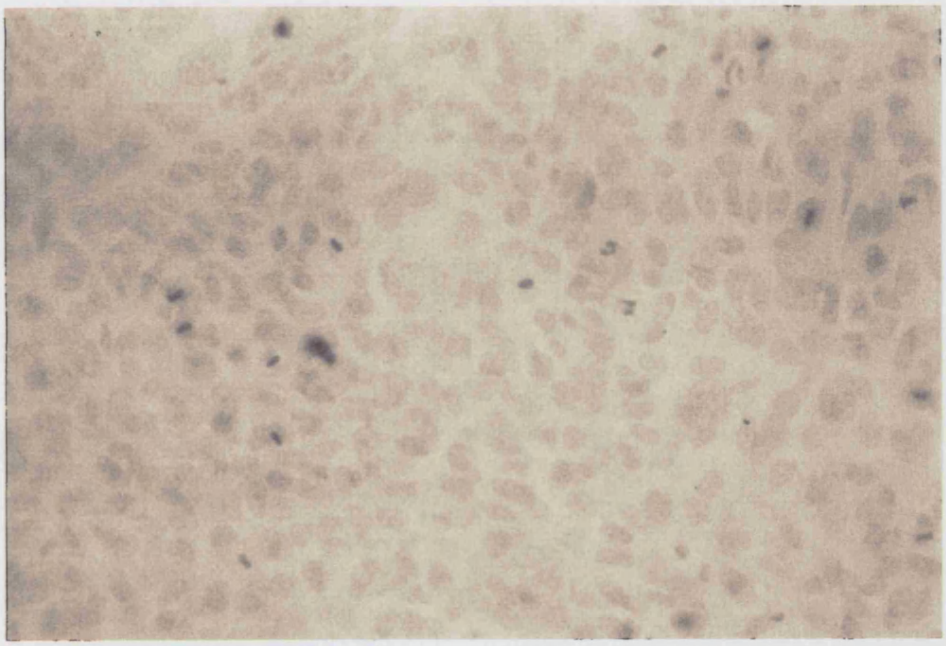

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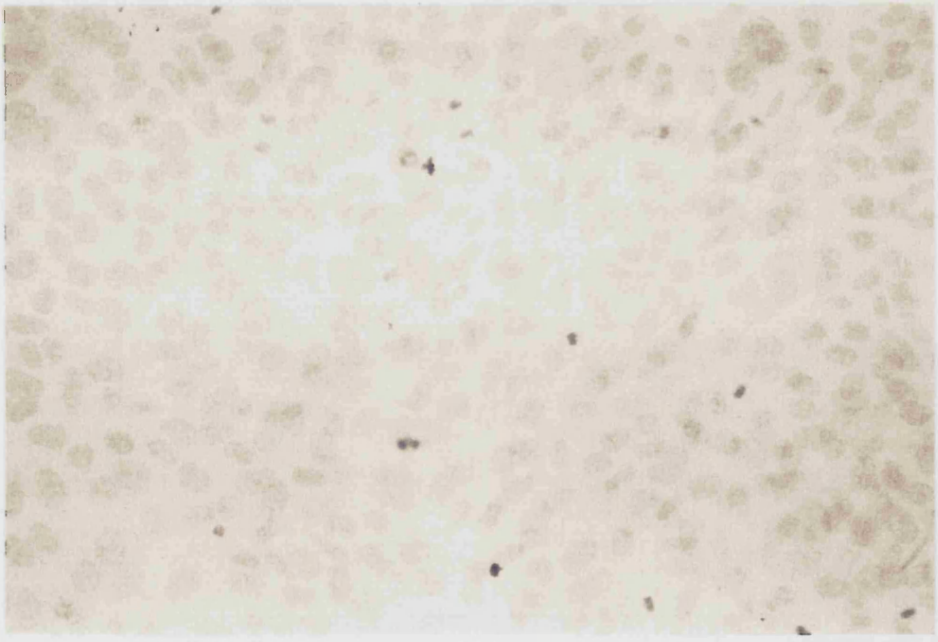
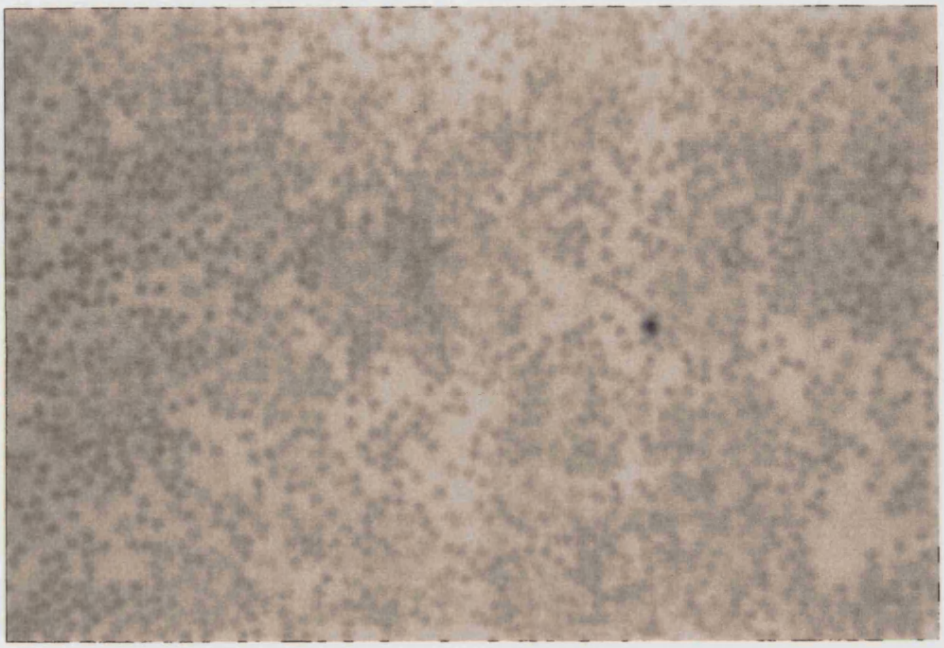


Figure 4.10 Continued...

C. Control PBMC

(+)



(-)

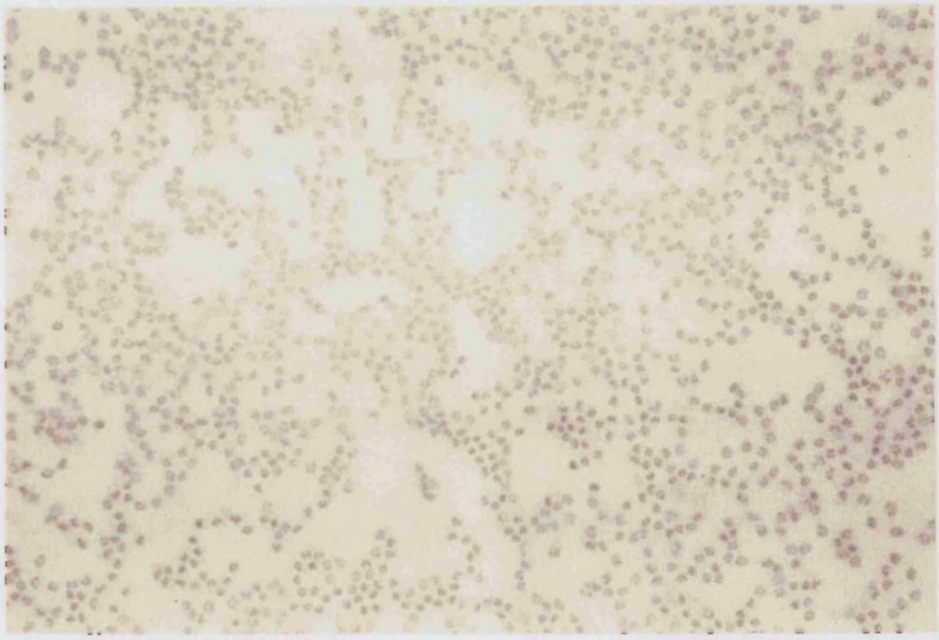


Figure 4.10 Continued...

D. RA PBMC

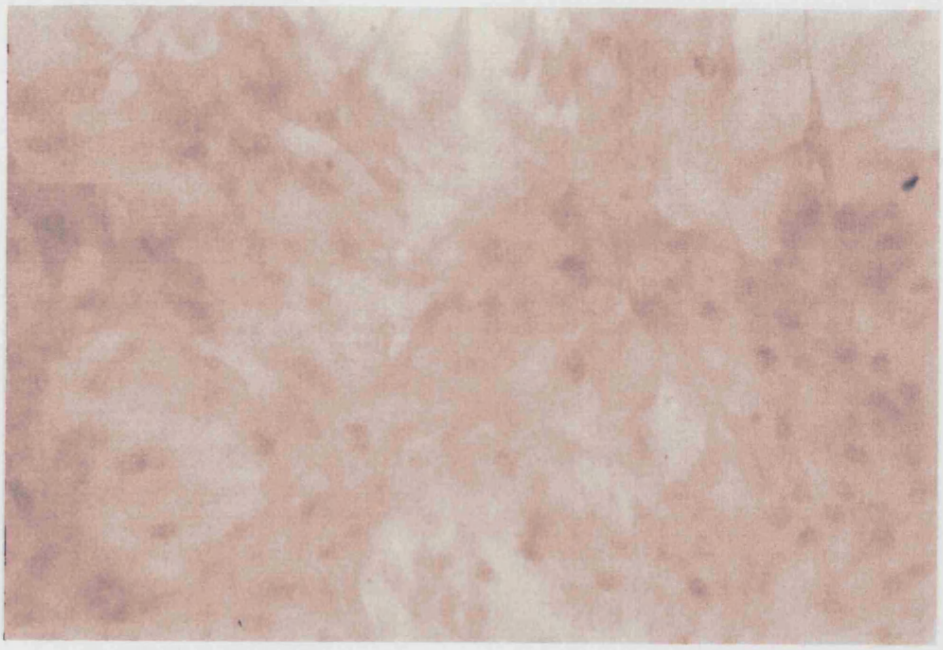
(+)

(-)

Figure 4.10 Continued...

E. Synovial fibroblasts

(+)



(-)

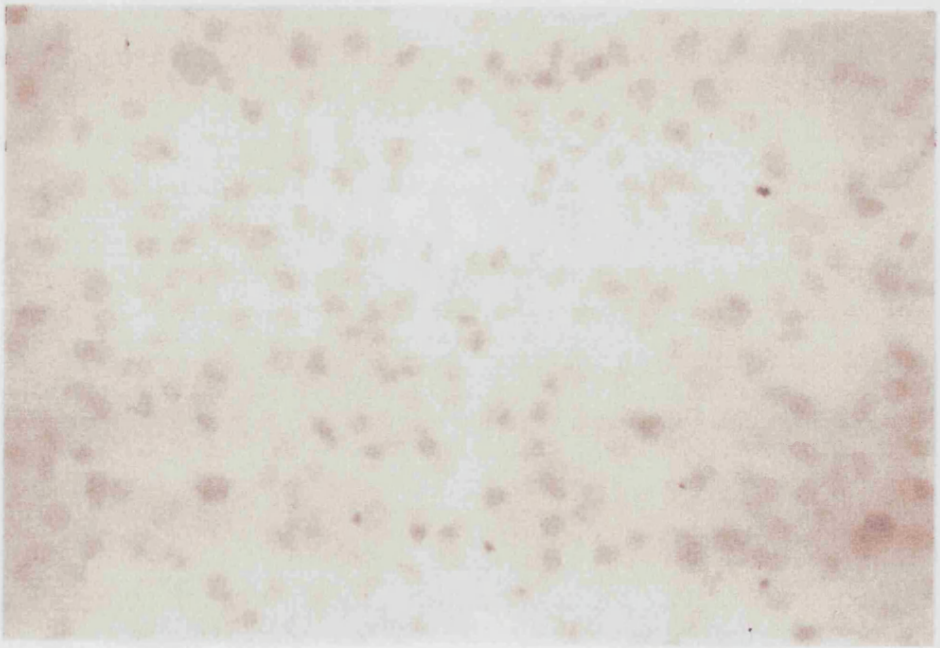
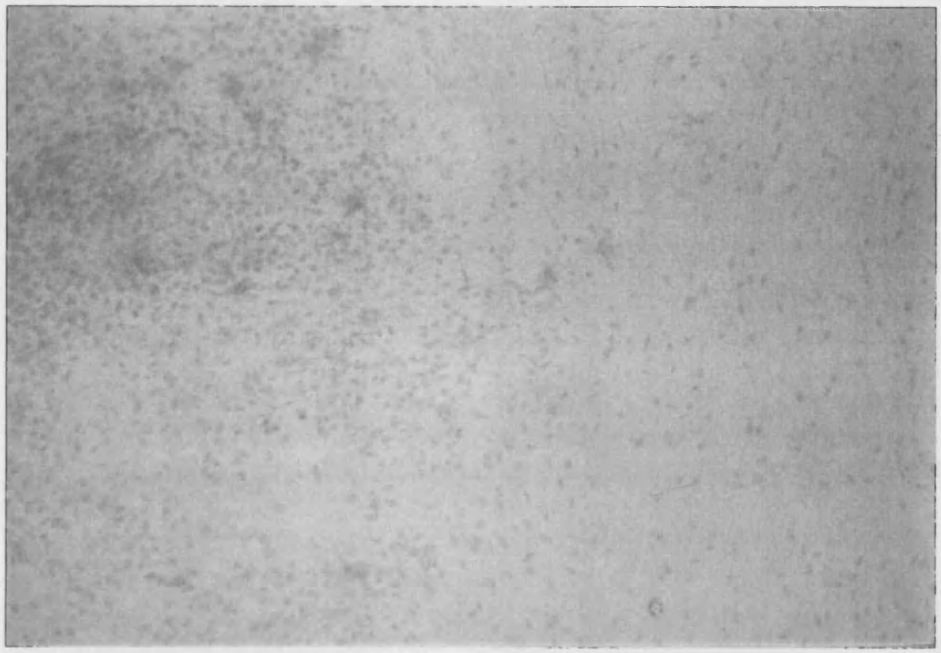


Figure 4.10 Continued...

F. RA synovial tissue

(+)



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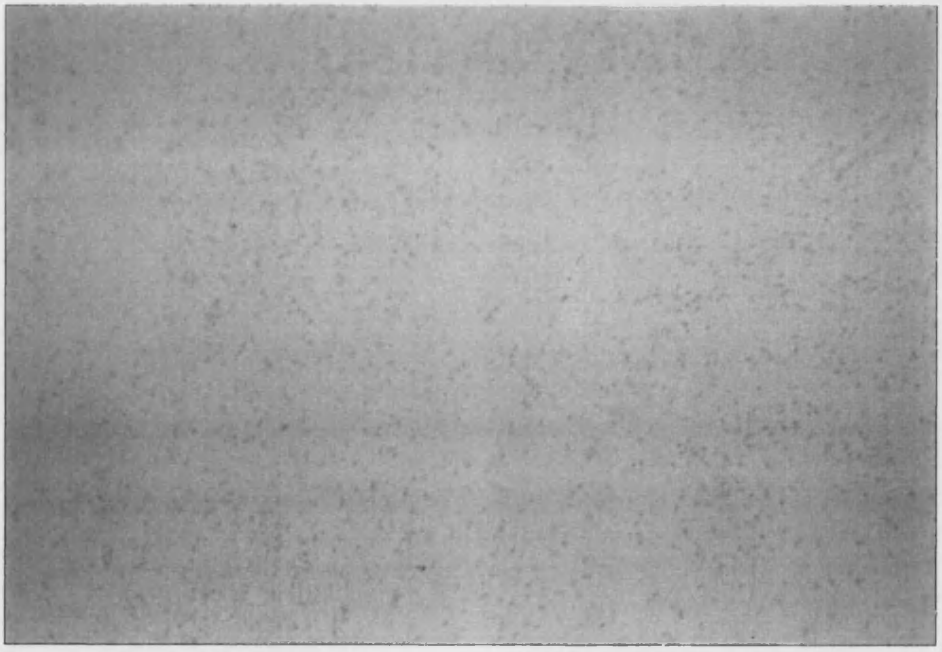


Figure 4.10 Detection of p29 antigen in cells and synovial sections

ZR-75 cells (A), Hs578T cells (B), PBMC from a control (C) and an RA patient (D), synovial fibroblasts (E) and RA synovial tissue (F) were stained with ER-D5 mAb, directed against p29 antigen (+), or a control antibody (-), using an APAAP procedure. (Original magnification X66 (A-E) and X33 (F)).

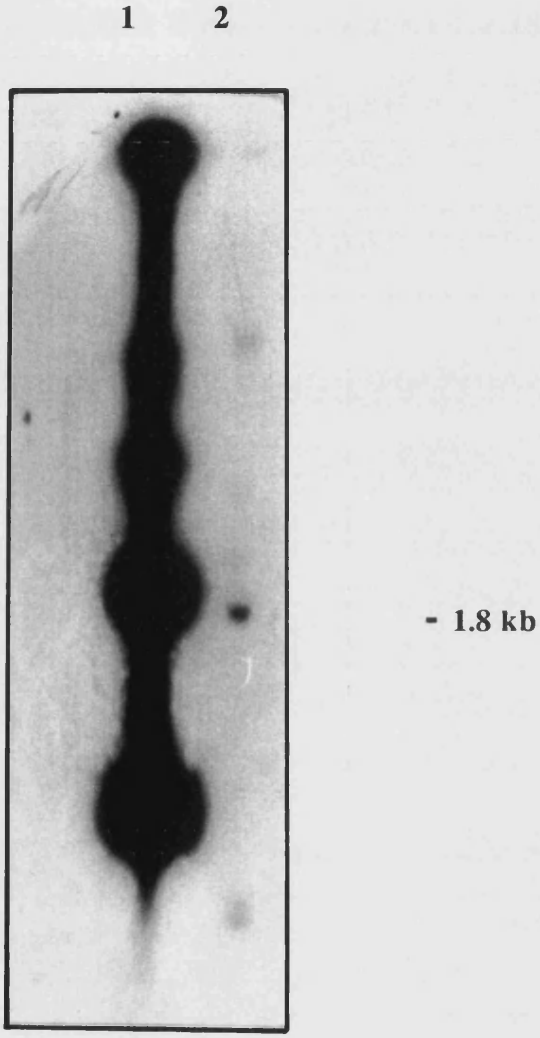


Figure 4.11 Southern blot analysis of oestrogen receptor cDNA

Oestrogen receptor plasmid DNA was Southern blotted and the filter was hybridised to cDNA obtained from the same plasmid, labelled as a probe, and autoradiographed. Lane 1 represents λHE marker and lane 2 represents the oestrogen receptor plasmid DNA.

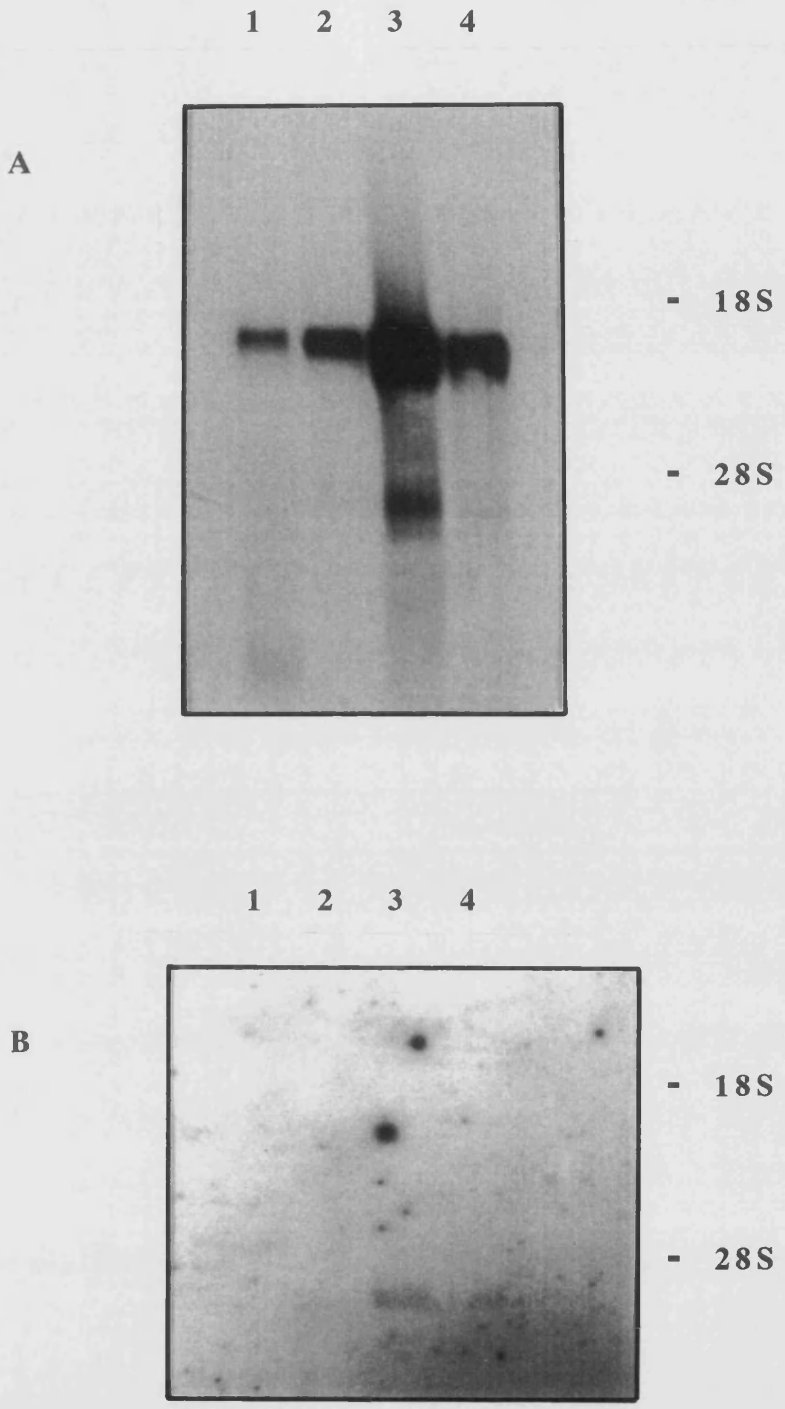


Figure 4.12 Detection of oestrogen receptor mRNA by Northern blot analysis

Total cell RNA was obtained from control PBMC (lane 1), Hs578T cells (lane 2), ZR-75 cells (lane 3) or T-47D cells (lane 4). 10 μg RNA was loaded onto each lane and Northern blotted. The filter was hybridised to GAPDH (A) and ER (B) cDNA probes and autoradiographed.

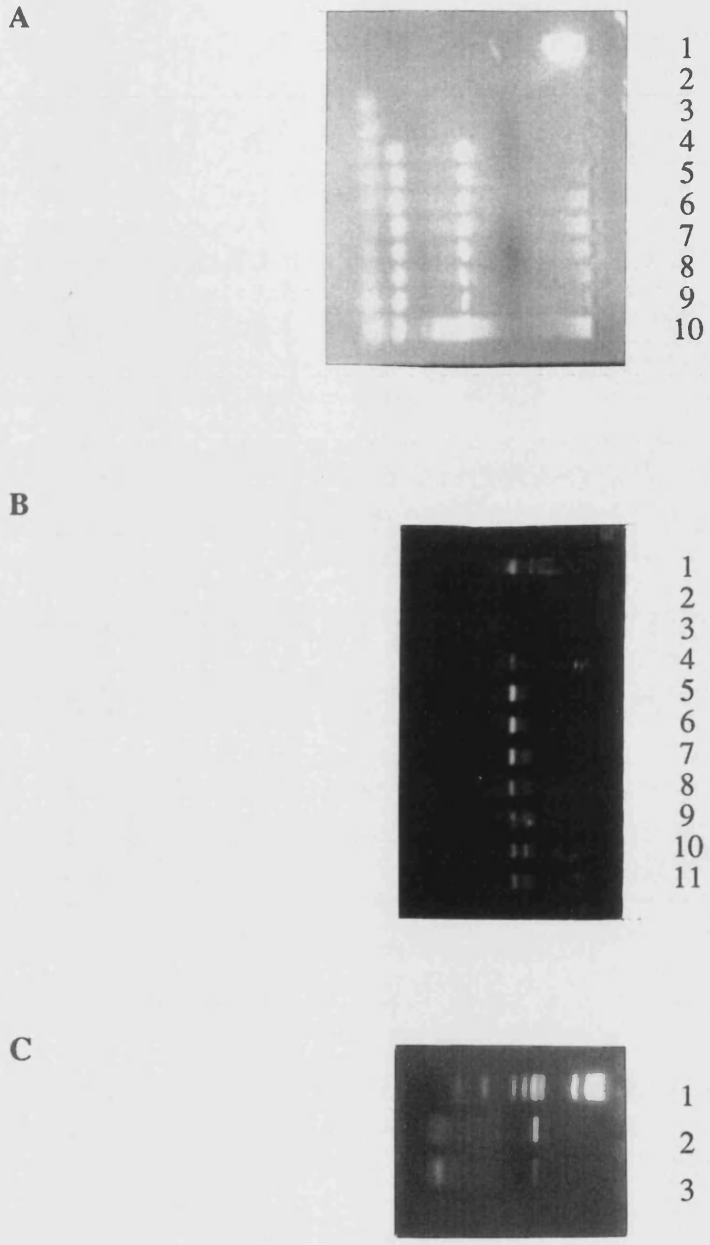


Figure 4.13 Optimisation of conditions for the RT-PCR

Total cell RNA was obtained from ZR-75 cells and (C) reverse transcribed using either Superscript (lane 2) or M-MLV (lane 3) reverse transcriptase, then amplified in a PCR using specific oligonucleotide primers designed from the published sequence of the human ER cDNA to flank a region within the DNA-binding domain. Reverse transcribed ZR-75 cell RNA was amplified with either (A) Promega or (B) Perkin-Elmer Taq polymerase with $MgCl_2$ concentrations ranging from 0-4 mM ((A) lanes 2-10) or 0-6 mM ((B) lanes 2-11), using ER-specific primers. 10 µl of the PCR reaction was run on each lane against Hae III digested ΦX-174-RF DNA marker ((A), (B) and (C) lane 1).

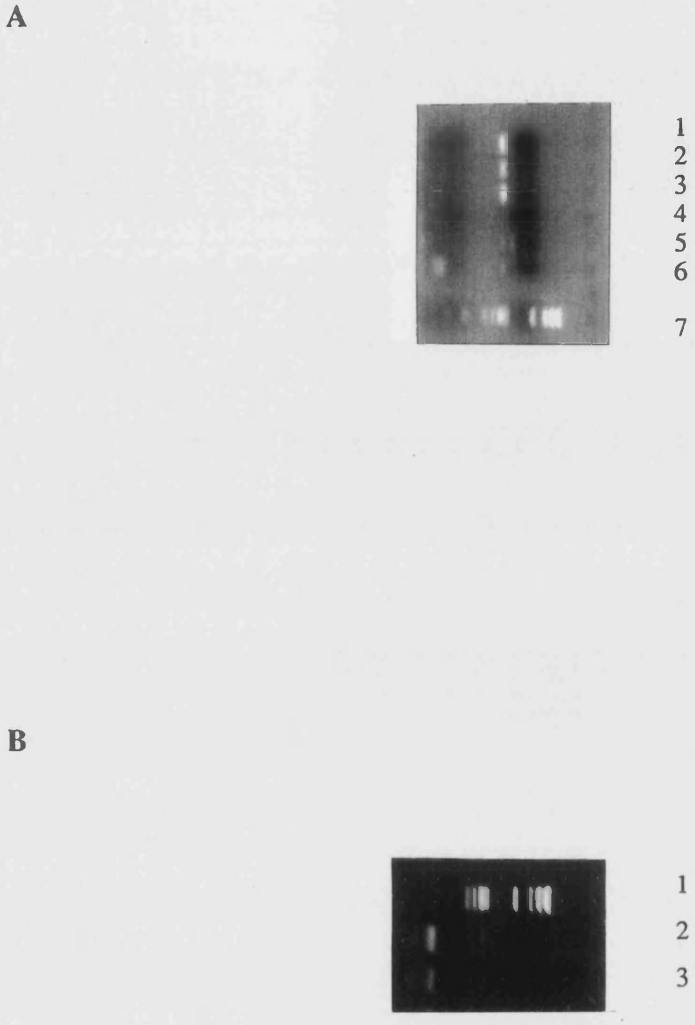


Figure 4.14 Detection of oestrogen receptor mRNA by RT-PCR

Total cell RNA was obtained from (A) ZR-75 cells (lanes 1-3), Hs578T cells (lane 4), control PBMC (lane 5) and T-47D cells (lane 6), and from (B) synovial fibroblasts (lanes 2 and 3). RNA was reverse transcribed then amplified in a PCR using specific oligonucleotide primers designed from the published sequence of the human oestrogen receptor cDNA to flank a region containing the DNA-binding domain. 10 µl of the PCR reaction was run on each lane against Hae III digested ΦX-174-RF DNA marker ((A) lane 7 and (B) lane 1).

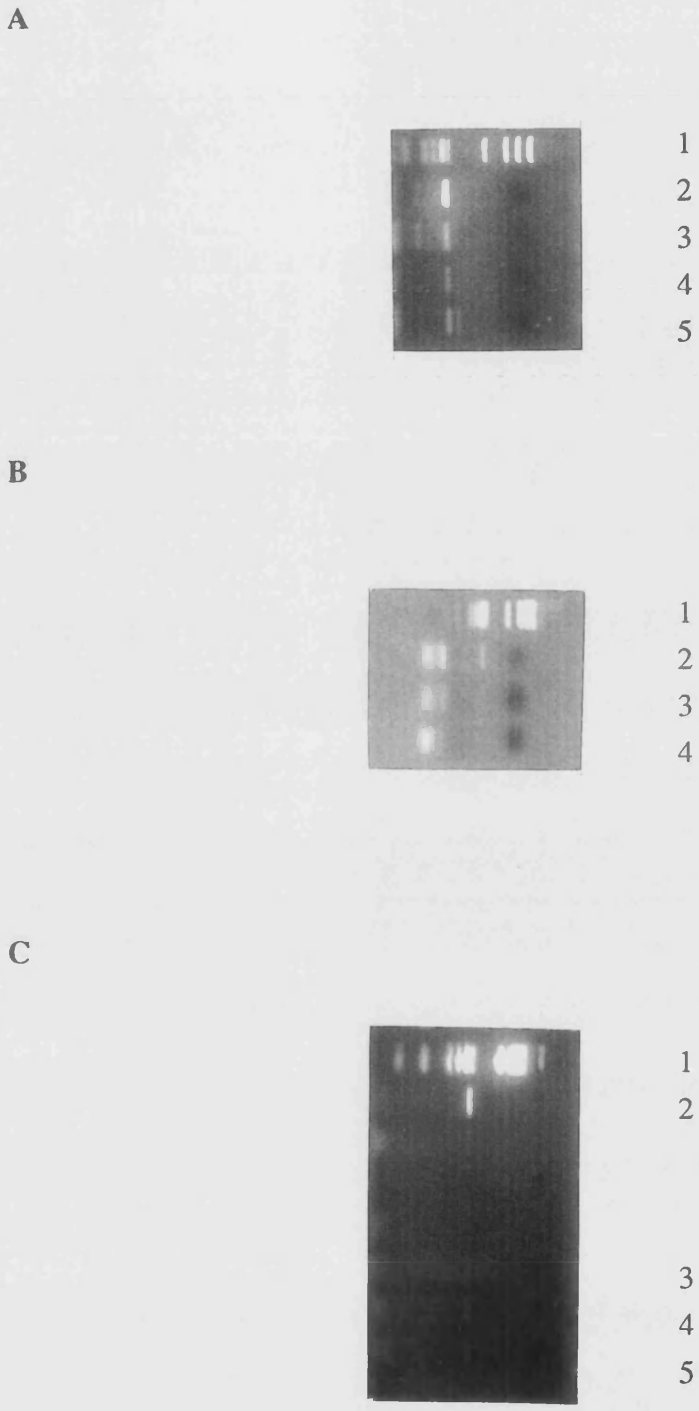


Figure 4.15 Detection of PBMC oestrogen receptor mRNA by RT-PCR

Total cell RNA was prepared from PBMC obtained from (A) premenopausal female controls (lanes 3-5), (B) male controls (lanes 2-4) and (C) perimenopausal (lane 4) and postmenopausal (lane 5) female and male (lane 3) RA patients. RNA was reverse transcribed then amplified in a PCR using specific oligonucleotide primers designed from the published sequence of the human oestrogen receptor cDNA to flank a region containing the DNA-binding domain. ZR-75 cell RNA was included as a positive control ((A) and (C) lane 2). 10 μl of the PCR reaction was run on each lane against Hae III digested ΦX-174-RF DNA marker ((A), (B) and (C) lane 1).

CHAPTER FIVE

SEX HORMONE MODULATION OF INTEGRIN EXPRESSION

5.1 INTRODUCTION

Adhesion molecules play a crucial role in mediating interactions between cells, and also between cells and the extracellular matrix, the integrins are one of three major families of adhesion molecules able to function in such interactions. The integrin superfamily exist as heterodimers, with non-covalently associated α- and β-subunits (reviewed by Springer, 1990; Hogg, 1991; Hynes, 1992). In RA it is thought that the infiltration and perpetuation of inflammatory cells in the joint is mediated, at least in part, by adhesion molecules such as the integrins (reviewed by Panayi, 1993). Moreover, certain of the receptors and their ligands have been shown to be upregulated on inflammatory cells and endothelial cells by inflammatory mediators, such as the cytokines IL-1 and TNF (reviewed by Pober & Cotran, 1990). This may contribute to the increase in cell migration into the synovium and synovial fluid. The upregulation of certain of the VLA integrins on infiltrating cells would also increase interactions with the extracellular matrix and with other cells (reviewed by Shimizu & Shaw, 1991) and hence contribute to the retention of these cells within the joint (see Introduction, Section 1.7).

In Chapter Four, ZR-75 cells were cultured in the presence and absence of oestrogen in order to assess the effect on ER expression. During the period of oestrogen-depletion a change in cellular growth and morphology was observed. Oestrogen is required to maintain ZR-75 cell growth under normal culture conditions and has been shown to induce proliferation of these cells (see Section 4.3.1). However, the alteration in morphology, in terms of loss of adherence, reduced granularity and colony formation, was unexpected. It was hypothesised that these changes, in particular the loss of adherence, might be due to a change in the level of certain adhesion molecules. As the VLA integrins are known to mediate cellular interactions with extracellular matrix components such as laminin, fibronectin and collagen (reviewed by Hogg, 1991; Hynes, 1992), the expression of these molecules, in terms of specific α- and β-subunits, was assessed in cells cultured in the absence and presence of oestrogen. For this purpose FACS analysis of cells incubated with a range of anti-integrin subunit mAbs was carried out. Integrin expression was examined at various time points after transferring the cells to oestrogen-depleted conditions, and also after reculturing the cells in oestrogen-supplemented medium following a period of oestrogen-deprivation.

Adhesion molecules play a crucial role in the immune system, both in mediating specific cellular interactions (reviewed by Springer, 1990), and in regulating the migration of immune cells within various microenvironments (reviewed by Shimizu & Shaw, 1991). In this respect, PBMC would be expected to express a wide range of

adhesion molecules, including several members of the integrin family. In addition, this spectrum of adhesion molecules would differ depending on the percentage of certain cell types within the PBMC population, as each cell type has its own array of such molecules which enable cell-specific interactions to occur. Therefore, apart from investigating which integrin subunits were expressed on resting control PBMC, T cells and monocytes were purified and incubated with the same range of mAbs, and the spectrum of integrin subunits expressed by the two cell populations ascertained by FACS analysis.

The observation that ZR-75 cell morphology was dependent on the presence of oestrogen and thus that, *prima facie*, sex hormones may be involved in the control of integrin expression, raised the question, was oestrogen able to differentially regulate integrin expression by PBMC in controls and RA patients? If so, then this would have important implications for the cellular interactions occurring in both the normal and pathogenic state. Oestrogen has been reported to modulate integrin expression in bone cells (Medhora *et al.*, 1991; Chiba *et al.*, 1992) and endothelial cells (Cid *et al.*, 1992). However, there are no such reports of a sex hormone effect on immune cell integrin expression. Therefore, experiments were designed to compare basal levels of the integrin subunits on PBMC from control and RA patients, and then to reassess the expression of the various subunits following culture of the cells with and without oestrogen. Results were analysed with respect to each individual subunit expressed by the cells and its role in mediating cell-cell and cell-extracellular matrix interactions.

5.2 METHODS

5.2.1 The Effect of Oestrogen-Depletion on ZR-75 Cell Morphology

ZR-75 cells were routinely cultured in RPMI supplemented with 10% (v/v) FCS and 10^{-9} M 17β-oestradiol (Method 2.4.3). To assess the effect of oestrogen-depletion on ZR-75 cell morphology, cells were transferred to phenol red-free RPMI supplemented with 10% (v/v) CS-FCS alone, or with 10^{-9} M 17α-oestradiol or 10^{-9} M 17β-oestradiol. Cells were cultured for up to 10 days, passaging when confluent (Method 2.4.4) but maintaining the oestrogen-depleted or oestrogen-supplemented culture conditions. Morphology was examined on a daily basis by phase-contrast microscopy.

To assess the recovery of ZR-75 cell morphology following oestrogen-depletion, cells were either maintained in phenol red-free RPMI supplemented with 10% (v/v) CS-FCS

and 10^{-9} M 17β-oestradiol or were cultured for 7 days in oestrogen-depleted conditions and then transferred into oestrogen-supplemented medium and cultured for a further 7 days. Cells were examined daily by phase-contrast microscopy.

5.2.2 The Effect of Oestrogen-Depletion on ZR-75 Cell Integrin Subunit Expression

Integrin subunit expression on ZR-75 cells was evaluated for cells maintained in normal culture conditions (Method 2.7.1). ZR-75 cells were then transferred to oestrogen-depleted or oestrogen-supplemented medium as described in Section 5.2.1. After 7 or 10 days, cells were harvested and incubated with antibodies directed against the integrin subunits known to be expressed under normal culture conditions, then examined by FACS analysis. Data were collected as % positive cells or MFI, with the latter being converted to sites per cell (Method 2.7.3). In addition, a time course was carried out by culturing ZR-75 cells with and without oestrogen and observing changes in integrin expression at 1, 3 and 7 days, as before. All cells were then transferred back into oestrogen-supplemented medium and the 'recovery' of integrin expression analysed, again at 1, 3 and 7 days. Comparisons for the time course of changes in integrin subunit expression and 'recovery' were made as sites per cell.

5.2.3 Analysis of Integrin Subunit Expression by T Cells and Monocytes

PBMC were prepared from peripheral blood of a normal female control (Method 2.4.1) and further purified as described (Method 2.4.2) to give T cell and monocyte cell populations. Cells obtained were analysed for their integrin subunit expression by incubating with mAbs directed against subunits previously shown to be expressed on resting PBMC from a normal female control, followed by incubation with an anti-IgG FITC-conjugate. T cells and monocytes were dual labelled with anti-CD3 or anti-CD14 R-phycoerythrin-conjugated antibodies, respectively (Method 2.7.1). Controls consisted of cells incubated with either the anti-integrin mAbs and anti-IgG FITC conjugate, or with the R-phycoerythrin antibodies, and a negative control was performed by incubating with second antibody, ie. fluorochrome, only. Data were obtained by FACS analysis by comparing the expression of FITC to that of R-phycoerythrin and hence determine the spectrum of integrin subunit expression for each of the cell populations.

5.2.4 The Effect of Oestrogen on Control and RA PBMC Integrin Subunit Expression

PBMC were prepared from peripheral blood obtained from male and female controls and RA patients (Method 2.4.1) and resuspended in phenol red-free RPMI supplemented with 10% (v/v) CS-FCS to a concentration of $1X10^6$ cells/ml. Cells were cultured in 10 cm^2 petri dishes with either a vehicle control, consisting of ethanol at a concentration equivalent to that of the highest hormone concentration, or 10^{-9} M 17β-oestradiol, for 48 hours. Cells were harvested by removing the supernatant and incubating with Versene for approximately 5 minutes, 4°C, to enable collection of the adherent cells from the surface of the petri dish. Adherent cells were combined with the supernatants and cells pelleted by centrifuging at 1000 rpm for 5 minutes. Integrin subunit expression was assessed by FACS analysis (Method 2.7.1), with data obtained as % positive cells or MFI, and the latter subsequently converted to sites per cell (Method 2.7.3).

5.3 RESULTS

5.3.1 The Effect of Oestrogen on ZR-75 Cell Morphology

ZR-75 cells were transferred from their normal oestrogen-supplemented culture conditions to an oestrogen-depleted environment and observed over a period of 10 days. The change from RPMI supplemented with 10% (v/v) FCS to phenol red-free RPMI supplemented with 10% (v/v) CS-FCS itself did not alter cell growth and morphology, as can be seen in Figure 5.1 by comparing ZR-75 cells under normal culture conditions (A), to ZR-75 cells grown for 7 days in the oestrogen-depleted medium, but with 10^{-9} M 17β-oestradiol added (B). 10^{-9} M is the concentration of 17β-oestradiol routinely added to ZR-75 cell cultures to maintain cell growth, and previous experiments have demonstrated that this produces sub-maximal cell proliferation (see Section 4.4.1). 10^{-9} M 17α-oestradiol also maintained cell integrity over a 7 day period (Figure 5.1C). However, when oestradiol was omitted from the culture medium for a total of 10 days, there was an inhibition of ZR-75 cell growth and the cells were seen to become less adherent (Figure 5.1D). In contrast to the cells maintained in oestrogen-supplemented culture conditions which formed large granular colonies and rapidly became confluent, cells grown in oestrogen-depleted medium became less granular and less adherent, formed only small colonies or existed as single cells in suspension, and

hence did not reach confluence. The change in morphology was gradual with a difference noted at day 7. These changes appeared to be maximal by day 10.

A more detailed time-course demonstrated that at day 1 both cell cultures exhibited similar morphology with cells settling after passaging (Figure 5.2A). ZR-75 cells which were grown under oestrogen-depleted conditions showed little change in growth or morphology at day 3 (Figure 5.2B (-)). By day 7, there was a certain degree of colony formation, but many of the cells were non-adherent and cell growth was noticeably reduced (Figure 5.2C (-)). In contrast, ZR-75 cells grown in phenol-red free RPMI supplemented with 10% (v/v) CS-FCS were virtually all adherent by day 3, with considerable cell growth and colony formation (Figure 5.2B (+)), and 7 days post-passaging the cells were virtually confluent (Figure 5.2C (+)).

To assess whether the change in ZR-75 cell growth and morphology following a 7 day period of oestrogen-depletion was reversible, cells were transferred to phenol red-free RPMI containing 10^{-9} M 17β-oestradiol, and compared to those cells which had been maintained in the latter medium throughout. After 1 day under these culture conditions there was little change in cell growth (Figure 5.3A (-/+)), whereas by day 3 almost all cells were adherent and there was a significant increase in growth and colonisation (Figure 5.3B (-/+)). The two cell populations were indistinguishable by day 7 (Figure 5.3C), with the ZR-75 cells transferred from oestrogen-depleted to oestrogen-supplemented conditions having almost reached confluence.

5.3.2 FACS Analysis of ZR-75 Cell Integrin Subunit Expression

A possible explanation for the alteration in ZR-75 cell morphology and the loss of adherence, as a result of oestrogen-depletion, was that the culture conditions determined integrin subunit expression by the cells. Therefore, ZR-75 cells were screened with a range of anti-integrin subunit antibodies, to determine which subunits were being expressed by the cells maintained under normal culture conditions, prior to investigating whether this expression was altered during oestrogen-deprivation. Cells were examined by FACS analysis, first selecting a homogeneous cell population (see Section 4.4.4), and the fluorescence obtained with the specific mAbs then compared to that of the control antibody. This illustrated that the subunits α2, α3, α6 and β1 were constitutively expressed on ZR-75 cells, whereas staining for α1, α4, α5 and αV subunits was negative (Figure 5.4). Titrations were made with each of the positively staining mAbs, to give saturating concentrations for use in subsequent experiments

(Figure 5.5). A 1/100 dilution was seen to give saturation of binding sites for each of the mAbs (Figure 5.5A-D).

5.3.3 The Effect of Oestrogen on ZR-75 Cell Integrin Subunit Expression

ZR-75 cells were cultured for 7 or 10 days in phenol red-free RPMI supplemented with 10% (v/v) CS-FCS alone, or with the addition of 10^{-9} M 17β-oestradiol. Integrin subunit expression was assessed for the two cell populations, as described above (Section 5.3.2). Results obtained for the oestrogen-depleted cells were expressed as a percentage of those for the cells maintained in the presence of 10^{-9} M 17β-oestradiol. As shown in Figure 5.6, depriving the cells of oestrogen resulted in a significant downregulation of α6 expression ($p<0.001$). In contrast, the expression of α2, α3 and β1 was unaltered. The downregulation occurred in terms of the number of ZR-75 cells expressing α6 (% positive cells) (Figure 5.6A) and the number of α6 sites per cell (Figure 5.6B). However, the maximal suppression in terms of % positive cells occurred at day 10, with levels reduced to 30% control (ie. 70% inhibition), compared to only 60% inhibition at day 7. In contrast, the depression of α6 sites per cell was maximal by day 7, with 75% inhibition of control levels, and this degree of inhibition was unchanged at day 10.

Further examination of the downregulation of ZR-75 cell α6 sites per cell in oestrogen-depleted culture conditions revealed that after only 1 day in culture without oestrogen there was a 30% inhibition, which was increased to 50% by day 3 and was maximal by day 7 at 60% (Figure 5.7A). After 7 days, the ZR-75 cells were transferred back to oestrogen-supplemented medium to assess the 'recovery' of α6, in terms of number of sites per cell of this integrin subunit. Figure 5.7B demonstrates that α6 expression returns to the normal level, ie. that seen for ZR-75 cells which had been maintained in medium supplemented with 10^{-9} M 17β-oestradiol, with the recovery being complete by day 7, although a certain degree of upregulation of α6 was seen at days 1 and 3. Preliminary data obtained suggested that incubation of ZR-75 cells with 10^{-10}M 17β-oestradiol and 10^{-6}M tamoxifen also resulted in downregulation of α6 expression, further indicating that the modulation of integrin expression was a direct result of oestrogen deprivation (results not shown).

The Hs578T cell line are ER-negative and hence oestrogen unresponsive. It was hypothesised that α6 expression may be constitutively lower in these cells, and

therefore a comparison was made between the ZR-75 and Hs578T cell lines, in terms of basal integrin expression. The results presented in Table 5.1 demonstrate that the Hs578T cell line has a different pattern of integrin expression, with all subunits tested shown to be positive. Whereas the expression of the α3 and β1 subunits was very similar between the two cell types, expression of α2 and α6 was considerably lower in the Hs578T cells.

Integrin subunit	Expression (sites per cell)	
	ZR-75 (n=6)	Hs578T (n=2)
α1	Negative	40921 (5590)
α2	376968 (78067)	17478 (862)
α3	205339 (36793)	207406 (32409)
α4	Negative	37894 (1100)
α5	Negative	17190 (2606)
α6	88355 (17391)	31688 (6480)
αV	Negative	N.D.
β1	596103 (98571)	475662 (51427)

Table 5.1 ZR-75 cell and Hs578T cell basal integrin subunit expression

ZR-75 and Hs578T cells were incubated with a range of antibodies directed against integrin subunits, diluted 1/50, or with an appropriate dilution of control antibody, followed by an anti-IgG FITC conjugate. Cells were examined by FACS analysis, with the data obtained as MFI which was subsequently converted to sites per cell. Results tabulated represent the means (SEM) of two or six separate experiments.

5.3.4 FACS Analysis of Control and RA PBMC Integrin Subunit Expression

Following the observations with ZR-75 cell integrin expression in the presence and absence of oestrogen, the effect of oestrogen on PBMC integrin subunit expression was examined, using PBMC from normal male and female controls and RA patients. As with the ZR-75 cells, initial experiments were performed to determine the integrin subunits expressed on resting cells, for which control PBMC were employed. This revealed significant expression of the subunits α4, α6, αL, αM, β1 and β2, whereas α2 and α3 were detectable at only very low levels and the staining for α1, α5 and β3 was essentially negative (Figure 5.8). Therefore, the former six were chosen for subsequent investigations. MAb titrations demonstrated that a 1/50 dilution of the antibodies was sufficient to produce saturation of binding sites (Figure 5.9).

In an attempt to gain more information as to the integrin subunit expression of specific cell types within the PBMC population, T cells and monocytes were negatively selected following incubation with specific antibodies directed against either monocytes and B cells, or T and B cells, respectively. Each cell population was then dual-labelled with the anti-integrin subunit mAbs described above and an antibody directed against the cell population of interest. Results were analysed as the percentage of cells expressing both fluorochromes compared to the percentage of cells expressing either FITC or R-phycoerythrin. Gates were initially set on the negative control which consisted of cells incubated with fluorochrome alone. T cells were shown to express all integrin subunits present on PBMC. The β2 and αL subunits were present on approximately 70-80% of T cells, β1, α4 and αM on approximately 50%, 40% and 25% cells, respectively, and α6 on only 10% of all T cells (Figure 5.10A). In contrast, β2 and αM subunits were detected on 75-85% of monocytes, with the expression of β1 and αL being 60-70% and the levels of the subunits α4 and α6 were negligible (Figure 5.10B).

5.3.5 The Effect of Oestrogen on Control and RA PBMC Integrin Subunit Expression

Prior to assessing whether control and RA PBMC respond differently to oestrogen, in terms of patterns of integrin expression, basal levels of α4, α6, αL, αM, β1 and β2 subunits were compared on control and RA PBMC. In addition, an anti-α5 mAb was included as there is some indication that the α4 and α5 integrins may be important in T cell accumulation within the joint in RA, via binding to fibronectin (Garcia-Vicuna *et*

al., 1992; Rodriguez *et al.*, 1992). Although in initial experiments screening PBMC for a range of integrin subunits, α5 was not expressed (Figure 5.8), the later studies showed that although the number of α5 binding sites per cell is low (Figure 5.11B), there are a significant number of positively staining cells (Figure 5.11A). When comparing control and RA cells, the β1 subunit was seen to be significantly lower on RA PBMC ($P<0.05$), in terms of % positive cells or sites per cell (Figure 5.11A and B), and the αL subunit was also lower on RA PBMC, but this was only significant when considering sites per cell ($p<0.05$) (Figure 5.11B). With the exception of the number of α6 sites per cell, all integrin subunits were constitutively lower on RA than control PBMC. The variable number of experiments included in Figure 5.11 was due to exclusion of the anti- α5 mAb in earlier experiments and the failure of α4 and αL antibodies to bind to cells in one particular experiment.

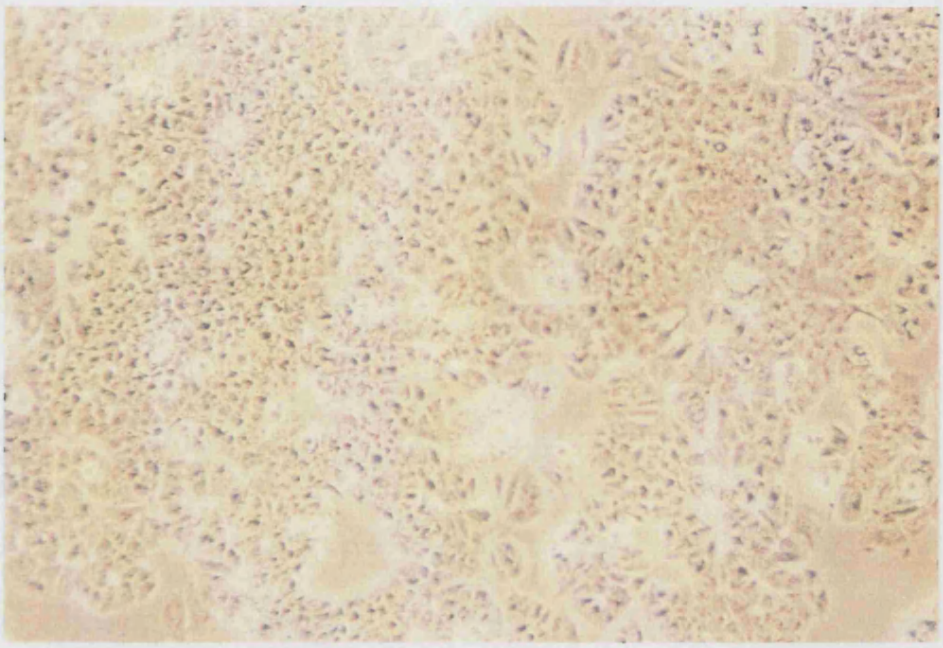
To evaluate the effect of oestrogen treatment on control and RA PBMC integrin expression, cells were cultured for 48 hours with or without 10^{-9}M 17β-oestradiol. Cells were then examined for expression of the integrin subunits shown to be present on resting control PBMC (see Section 5.3.4). For ZR-75 cells, the oestrogen-depleted population were considered to be the treatment group and the integrin expression of these cells was compared to that of the cells maintained in medium supplemented with 10^{-9}M 17β-oestradiol. However, as it was the effect of oestrogen on PBMC integrin expression which was under observation, results with the oestrogen-treated group were compared to those of the oestrogen-depleted group. Preliminary results obtained by comparing integrin expression on resting PBMC to the expression following 48 hours of culture suggested that α4, β1 and αL expression was increased on culturing cells, whereas αM expression was reduced slightly and α6 and β2 remained unchanged (results not shown). Figure 5.12 illustrates that integrin expression was essentially unchanged in the PBMC cultured with 10^{-9}M 17β-oestradiol, compared to cells cultured in medium alone. The αM subunit appeared to be significantly higher on RA than normal PBMC following oestrogen treatment ($p<0.05$) (Figure 5.12B). However, the low number of experiments included make these results somewhat tenuous.

5.4 SUMMARY

Results demonstrated that ZR-75 cells cultured under oestrogen-depleted conditions became less adherent, with a general reduction in cell growth and colony formation observed. This was reversible following reculture in oestrogen-supplemented medium. Changes in morphology were associated with a significant inhibition of α6 subunit

expression by ZR-75 cells, whereas the levels of the other subunits constitutively expressed remained unchanged. The subsequent reculturing of the cells in the presence of oestrogen was sufficient to cause the upregulation of α6 expression to levels comparable with those seen under normal culture conditions. PBMC expressed a different spectrum of integrin subunits to ZR-75 cells, and within the PBMC population, T cells and monocytes also differed in terms of levels of these subunits. Examination of the basal levels of the subunits on resting PBMC from normal controls and RA patients revealed a slight suppression in RA patients. However, oestrogen had no effect on the integrin expression of control or RA PBMC.

A. RPMI + 10% FCS + 17β-oestradiol



B. Phenol red-free RPMI + 10% CS-FCS + 17β-oestradiol

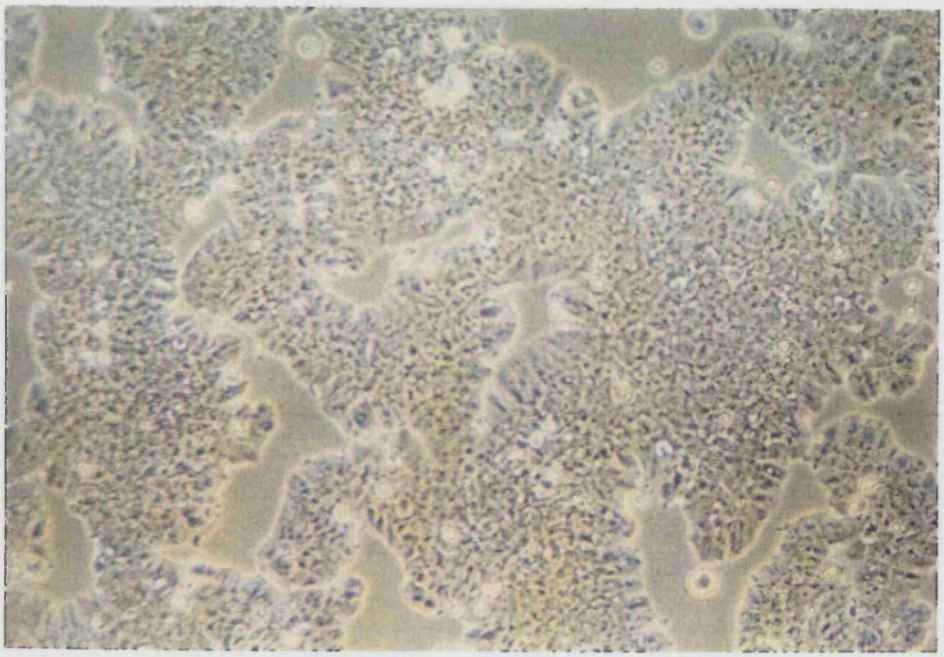
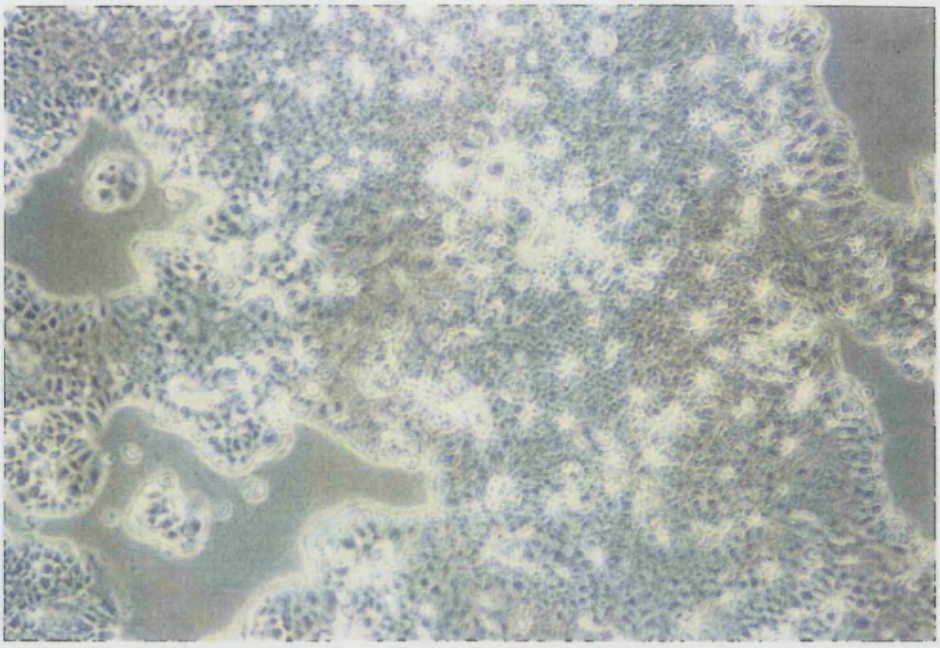


Figure 5.1 Continued...

C. Phenol red-free RPMI + 10% CS-FCS + 17α-oestradiol



D. Phenol red-free RPMI + 10% CS-FCS

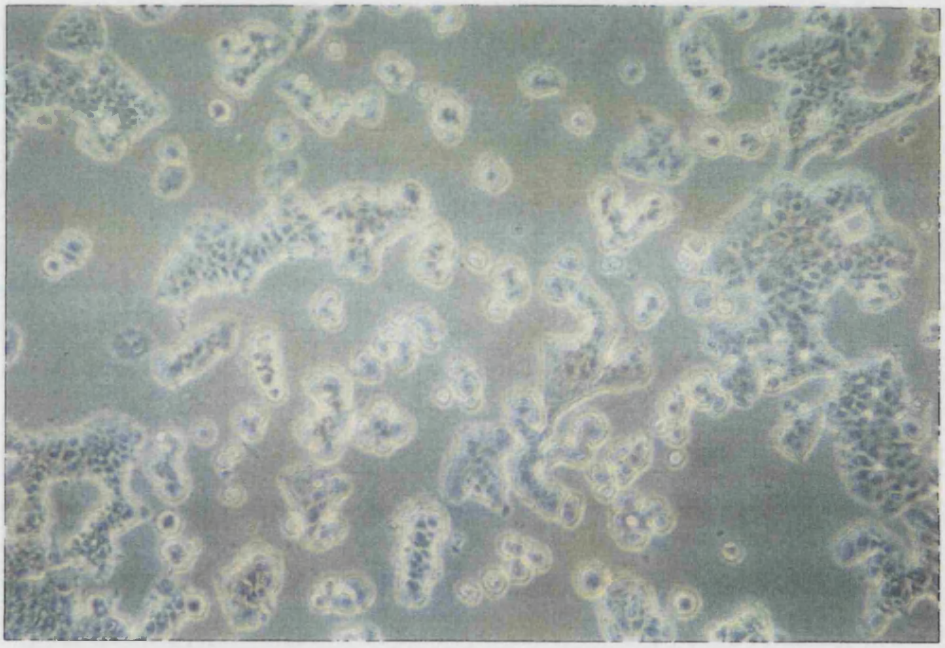
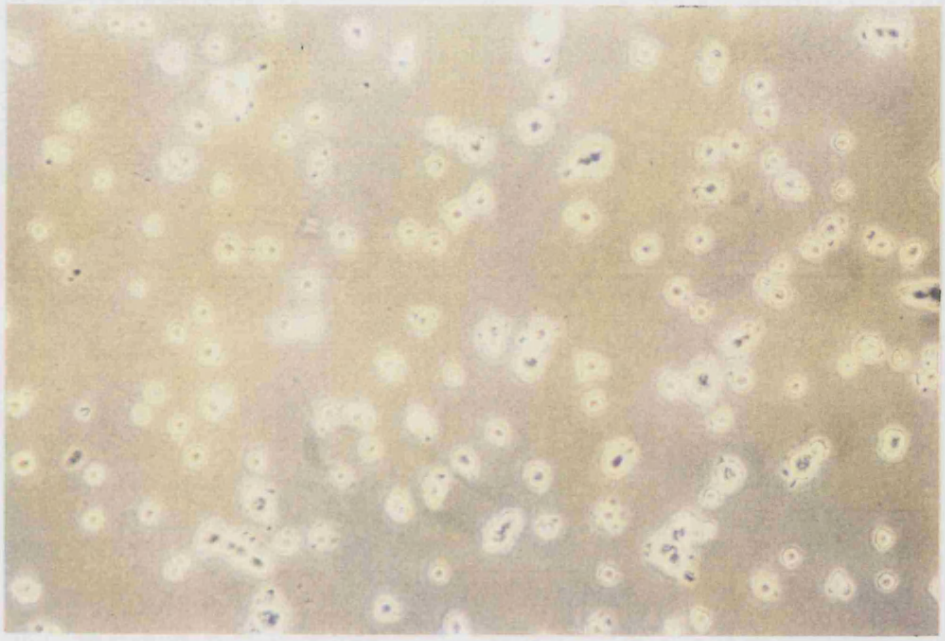


Figure 5.1 The effect of oestrogen on ZR-75 cell morphology

ZR-75 cells were cultured for 7 days in (A) RPMI supplemented with 10% FCS and 10^{-9} M 17β-oestradiol, or phenol-red free RPMI supplemented with 10% CS-FCS and 10^{-9} M 17β-oestradiol (B) or 10^{-9} M 17α-oestradiol (C), or in the absence of oestrogen (D). Cell morphology was observed by phase-contrast microscopy.

A. Day 1

(+)



(-)

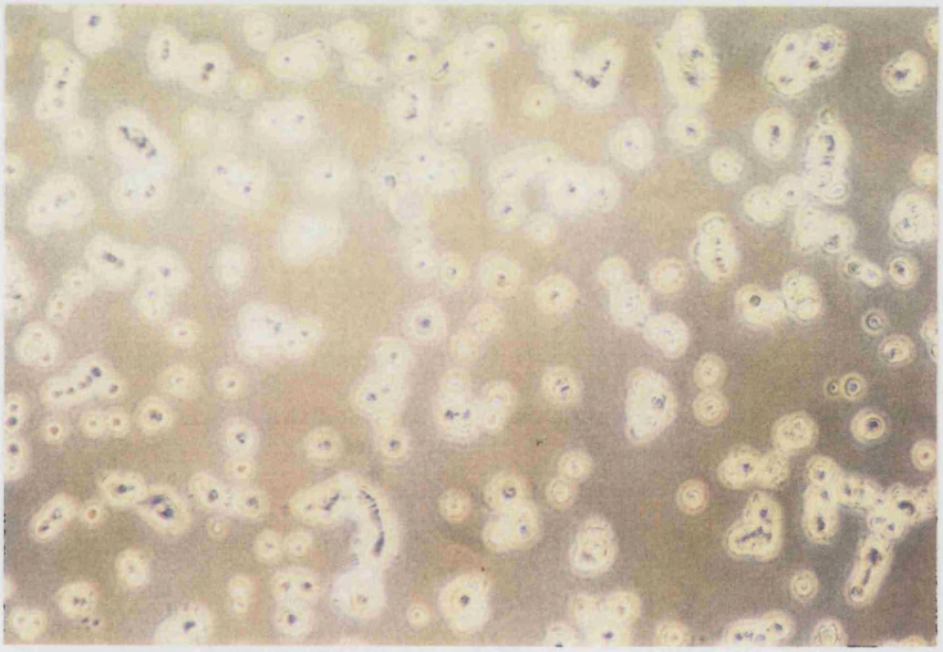
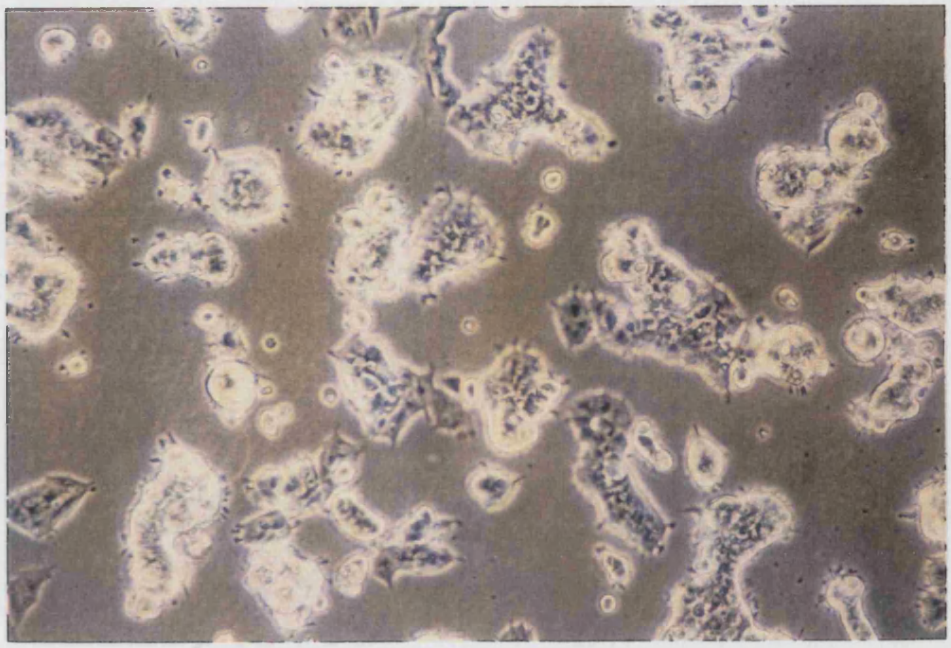


Figure 5.2 Continued...

B. Day 3

(+)



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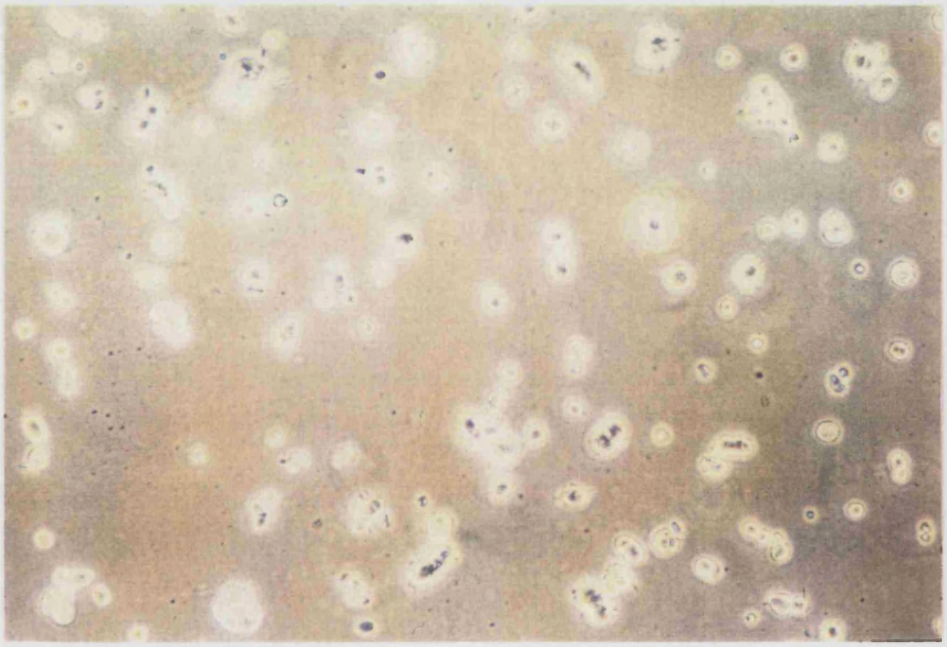
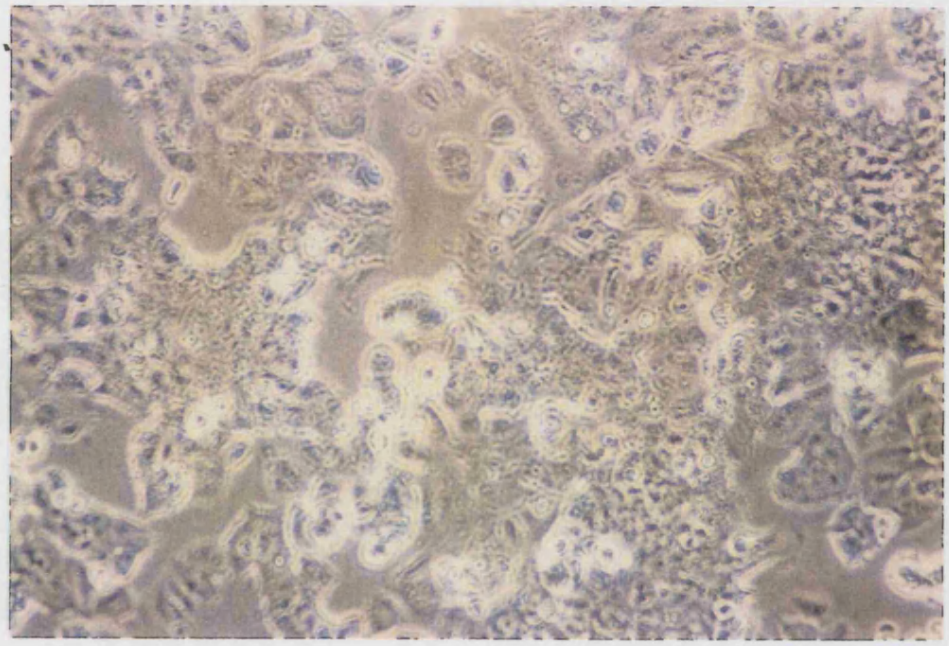


Figure 5.2 Continued...

C. Day 7

(+)



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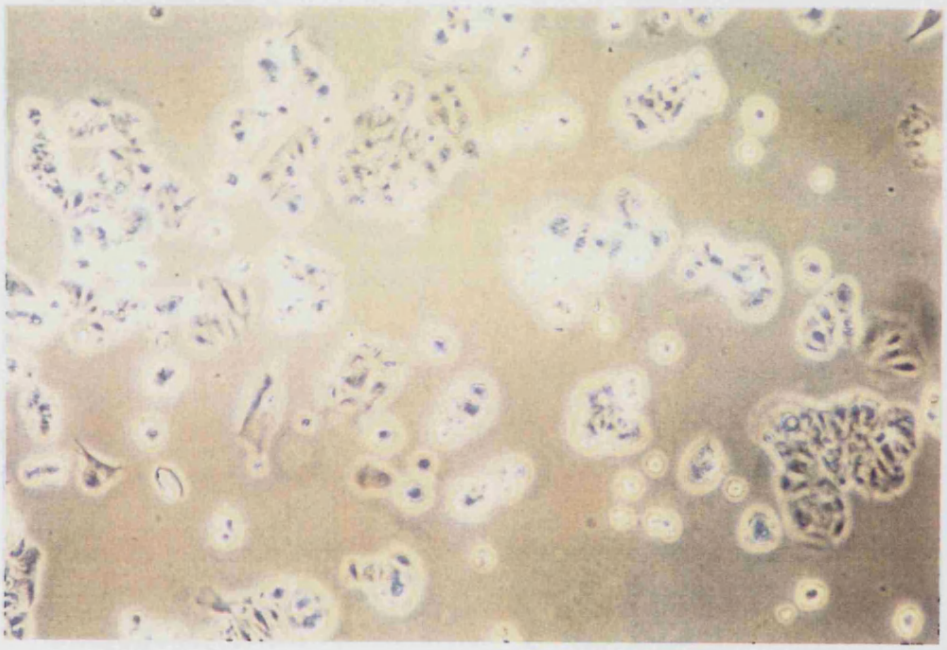
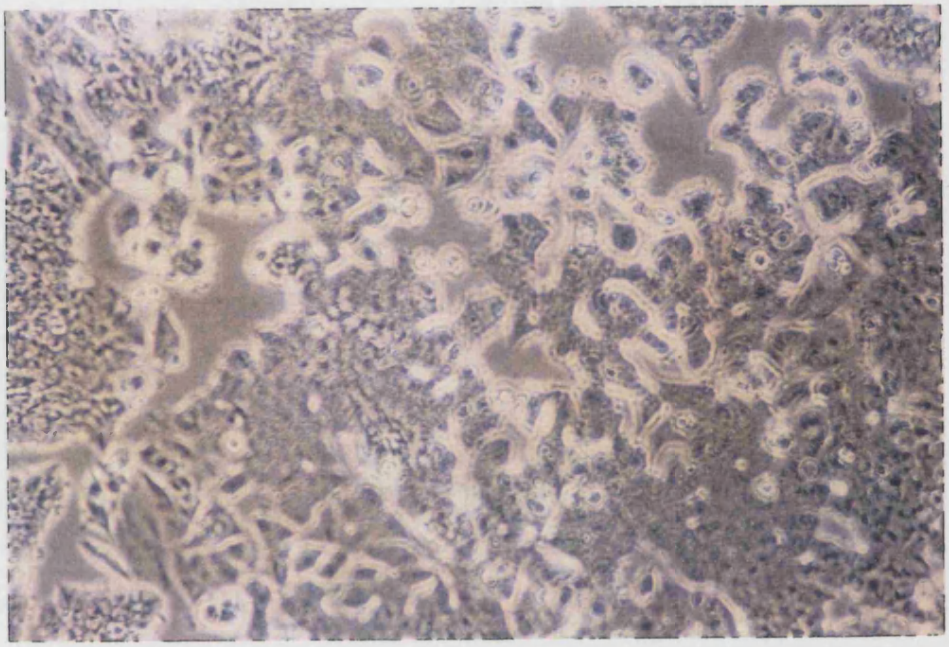


Figure 5.2 Time-course for modulation of ZR-75 cell morphology

ZR-75 cells were transferred from RPMI supplemented with 10% FCS and 10^{-9} M 17β-oestradiol to phenol-red free RPMI supplemented with 10% CS-FCS and were cultured for (A) 1 day, (B) 3 days or (C) 7 days in the presence (+) or absence (-) of 10^{-9} M 17β-oestradiol. Cell morphology was observed by phase-contrast microscopy.

A. Day 1

(+/+)



(-/+)

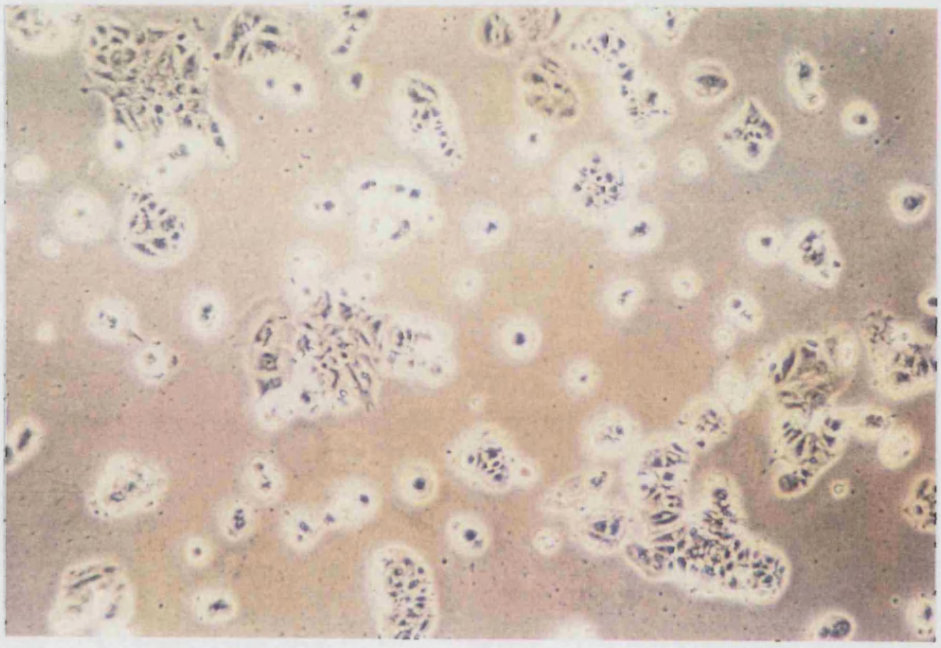
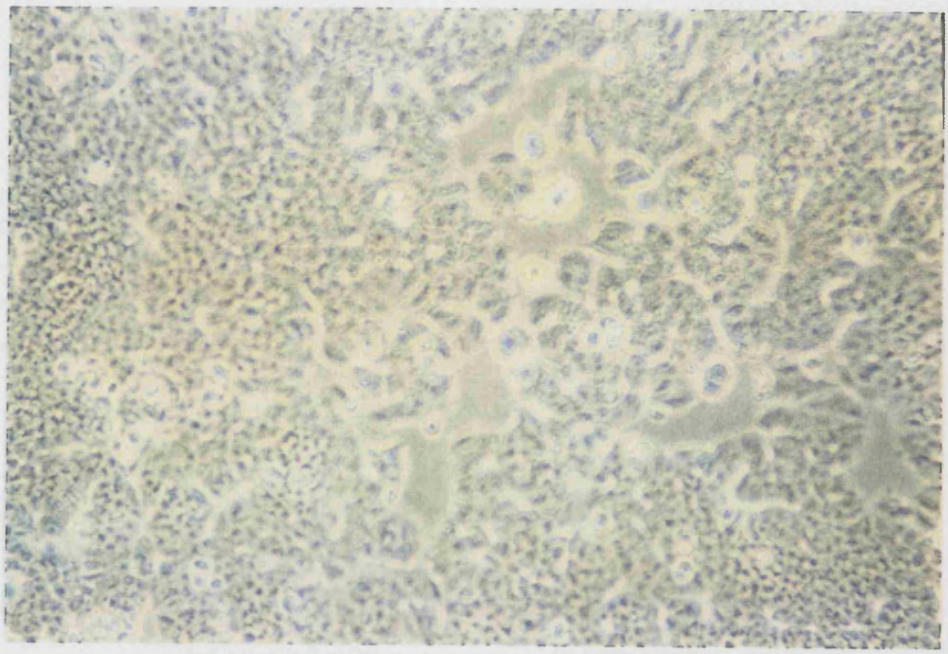


Figure 5.3 Continued...

B. Day 3

(+/+)



(-/+)

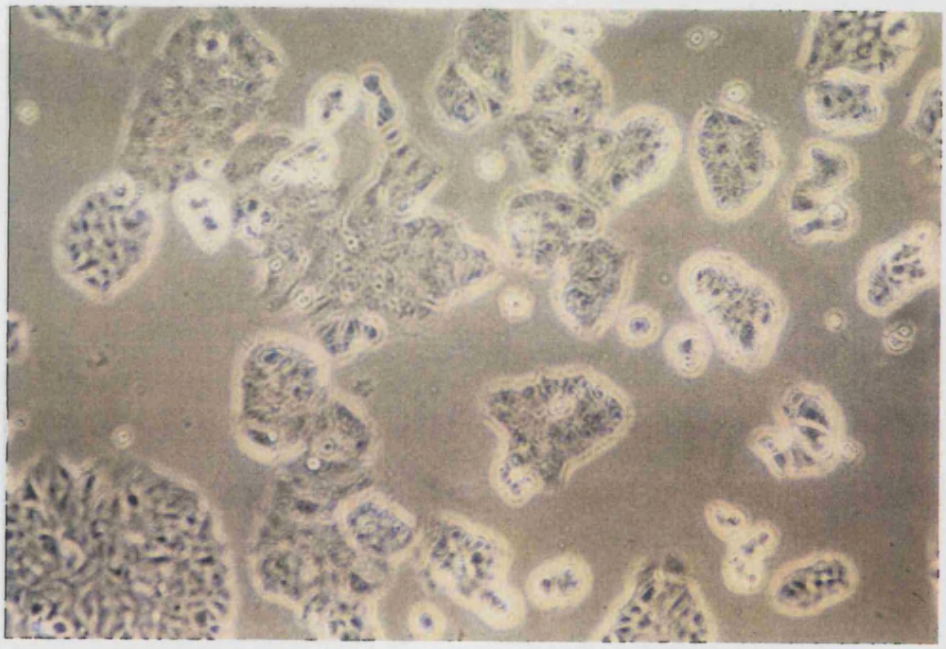
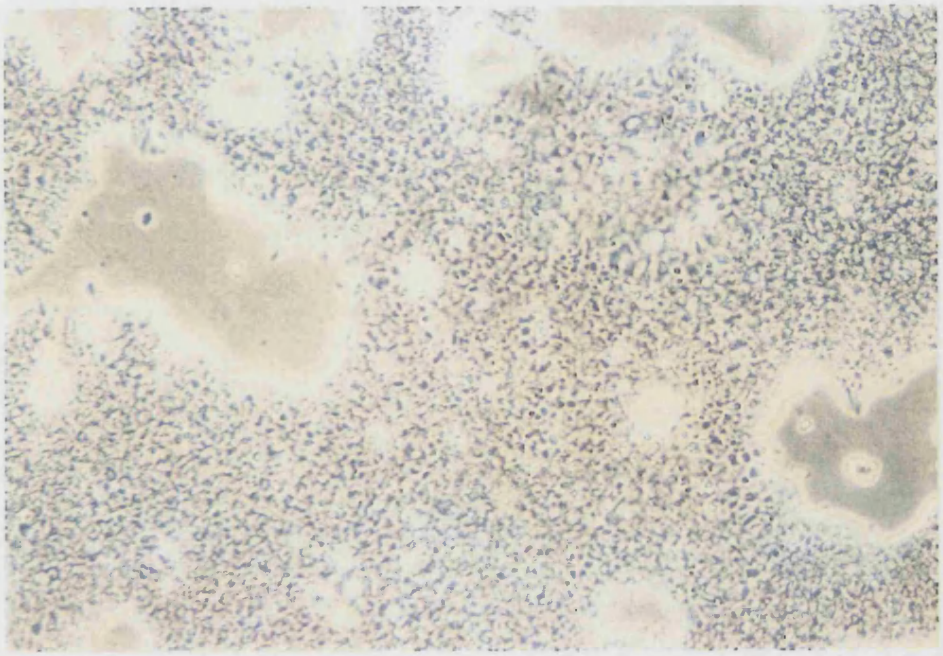


Figure 5.3 Continued...

C. Day 7

(+/+)



(-/+)

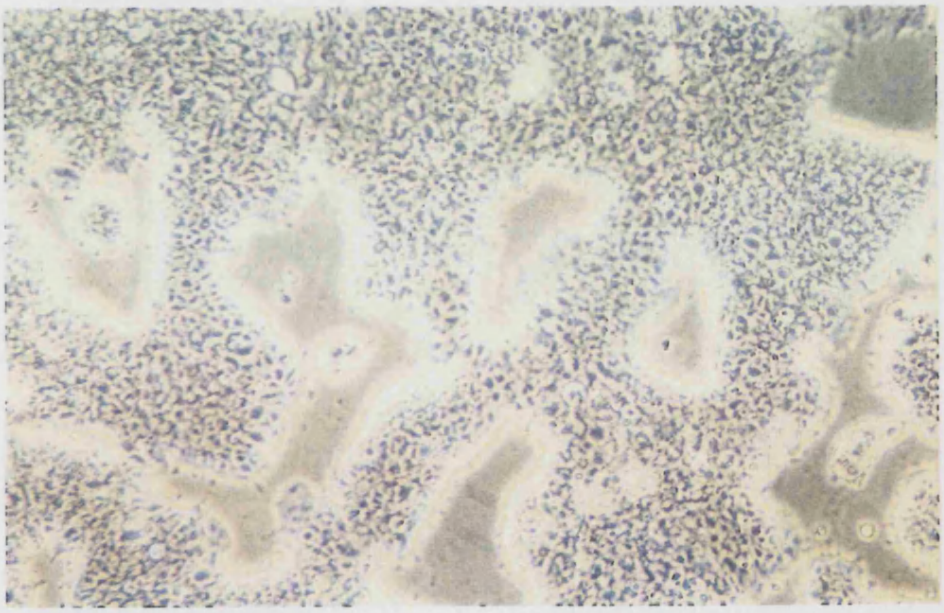


Figure 5.3 Recovery of ZR-75 cell morphology

ZR-75 cells were either maintained in phenol red-free RPMI supplemented with 10% CS-FCS and 10^{-9} M 17β-oestradiol (+/+), or were cultured for 7 days in oestrogen-depleted medium and then transferred to medium containing 10^{-9} M 17β-oestradiol (-/+) for (A) 1 day, (B) 3 days or (C) 7 days. Cell morphology was observed by phase-contrast microscopy.

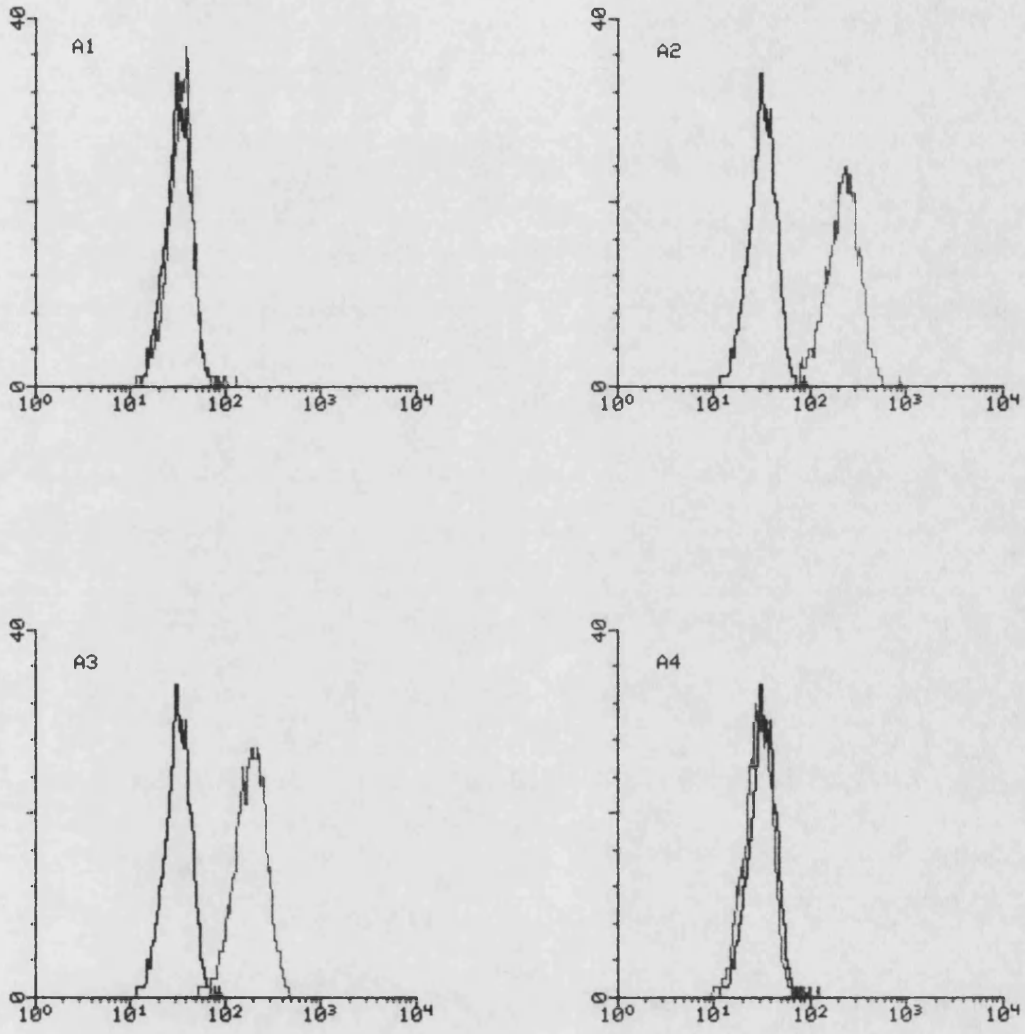


Figure 5.4 Continued...

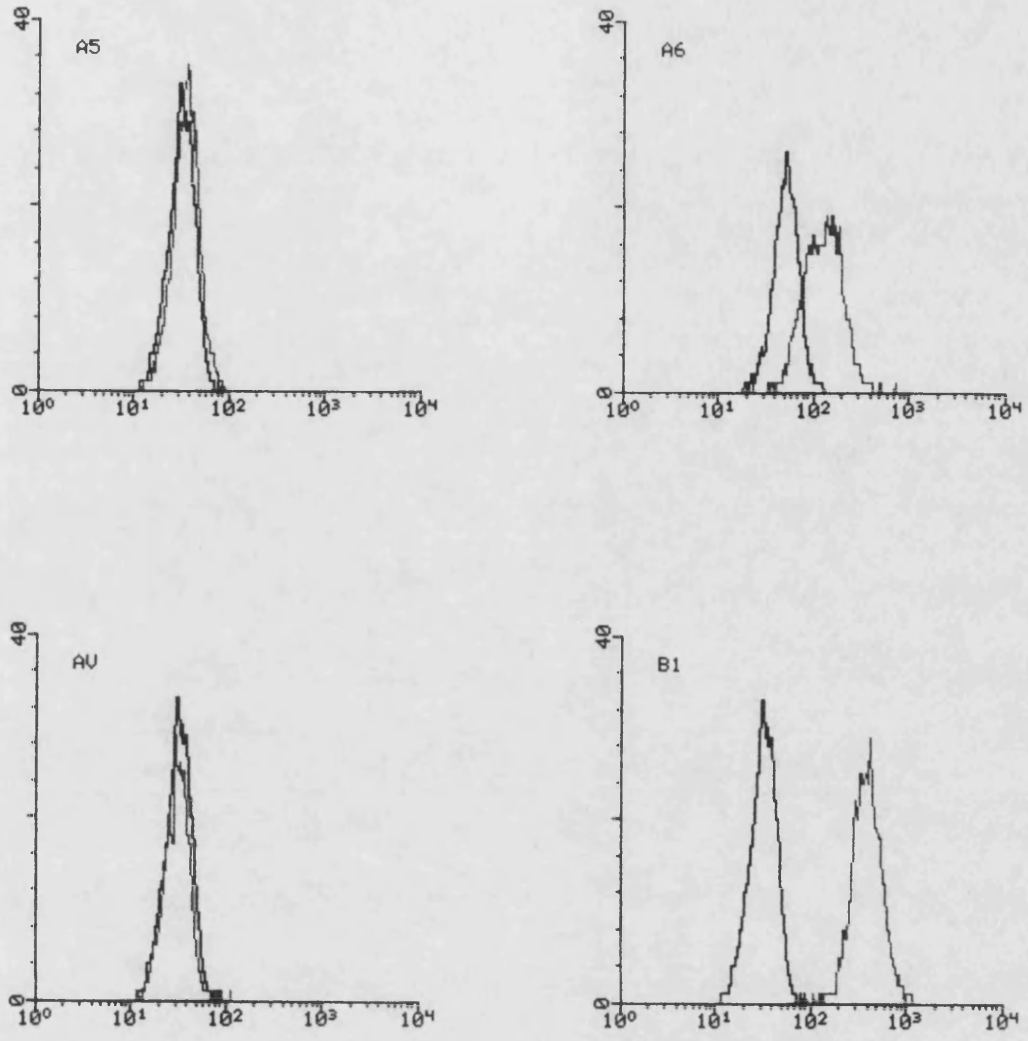


Figure 5.4 FACS analysis of ZR-75 cell integrin subunit expression

ZR-75 cells were incubated with mAbs directed against integrin subunits, all of which were diluted 1/50 except for the anti-αV mAb which was used neat, or with control IgG diluted to the appropriate concentration. Cells were then incubated with an anti-IgG FITC-conjugate and examined by FACS analysis. Results are expressed as histograms of fluorescence intensity.

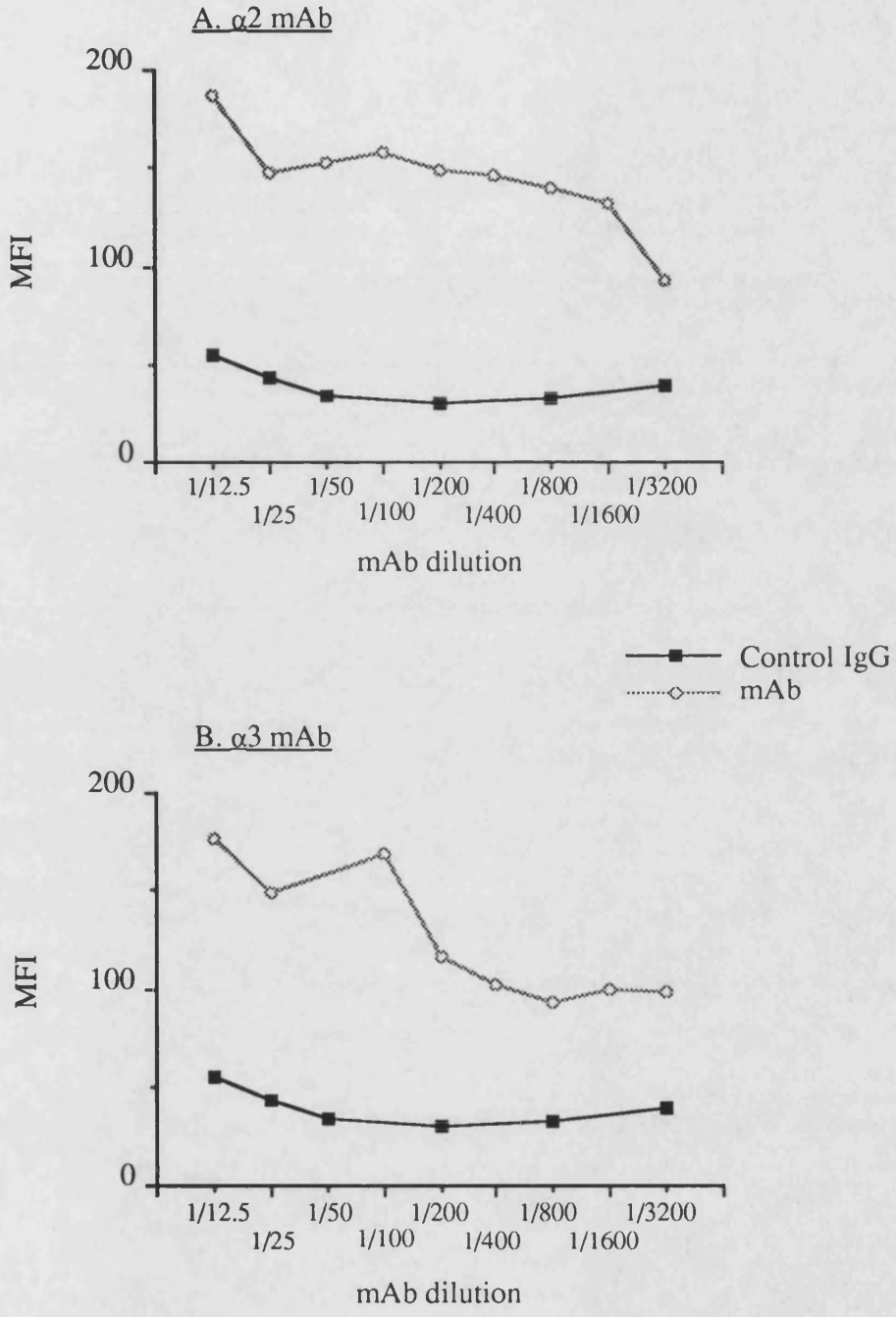


Figure 5.5 Continued...

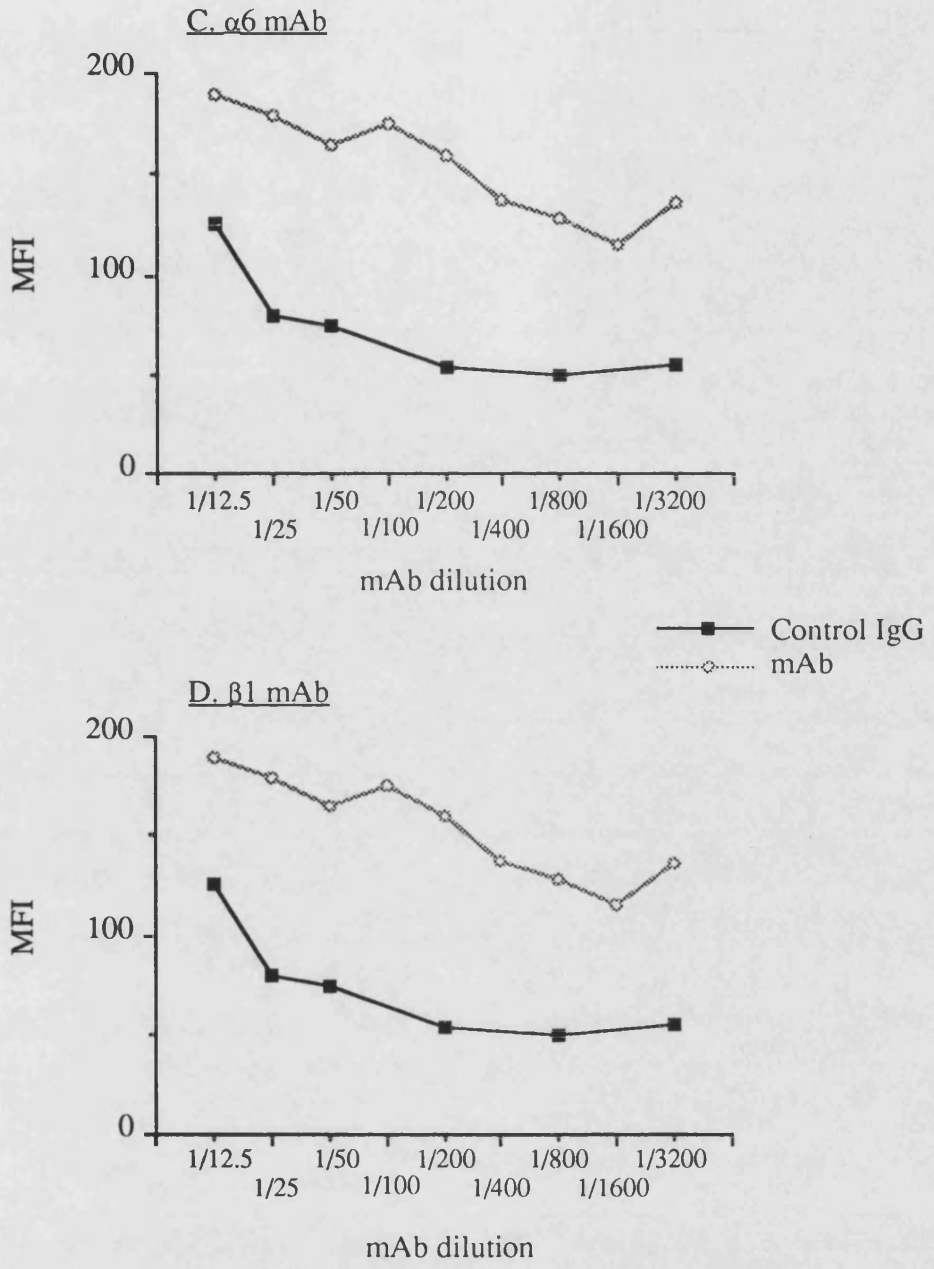


Figure 5.5 Titration of anti-integrin antibody binding to ZR-75 cells

ZR-75 cells were incubated with mAbs directed against the integrin subunits (A) α2, (B) α3, (C) α6 or (D) β1, or with control IgG diluted to the same concentrations. Cells were then incubated with an anti-IgG FITC conjugate and examined by FACS analysis. Results are expressed as mean fluorescence intensity (MFI) and plotted as a titration curve.

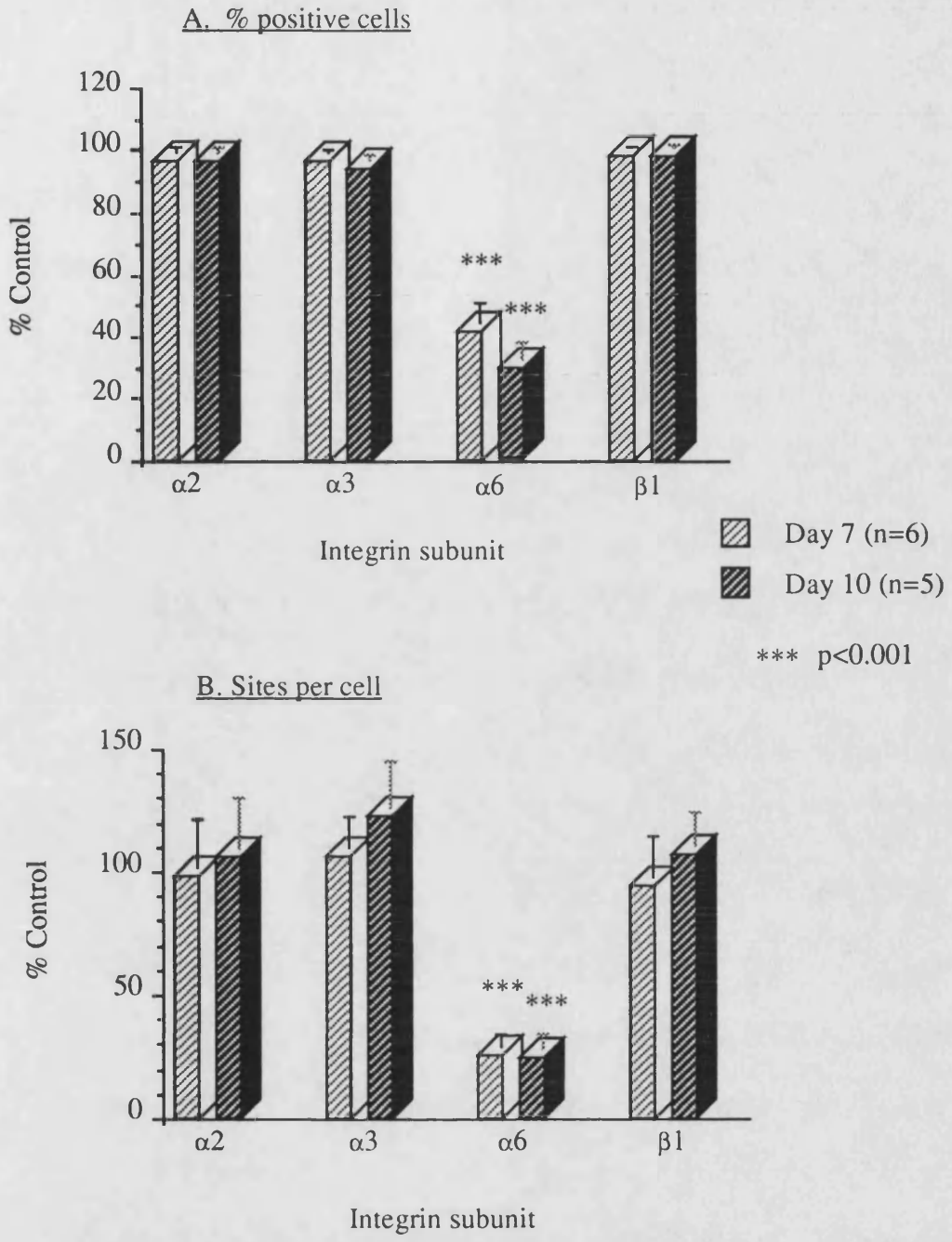
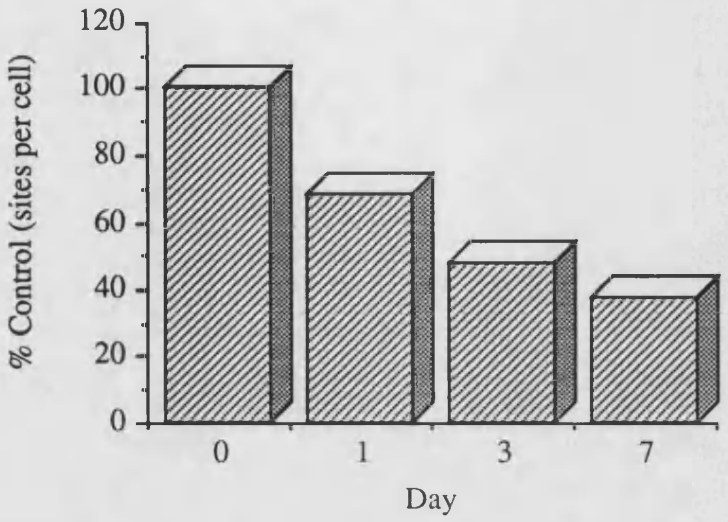


Figure 5.6 Oestrogen modulation of ZR-75 cell integrin subunit expression

ZR-75 cells were cultured in phenol red-free RPMI supplemented with 10% (v/v) CS-FCS in the presence or absence of 10^{-9} M 17β-oestradiol. After 7 or 10 days, cells were harvested and incubated with anti-α2, -α3, -α6 or -β1 mAbs at saturating concentrations, or with control IgG diluted to the same concentration. Cells were then incubated with an anti-IgG FITC-conjugate and examined by FACS analysis. Data was obtained as % positive cells relative to the control IgG which was set at 5% (A), or mean fluorescence intensity which was converted to sites per cell (B). Antibody dilutions was performed in triplicate and data obtained was combined to give an average value. Results are expressed as % control, ie. integrin expression of ZR-75 cells cultured in the presence of oestrogen. Each histogram represents the mean ± SEM of five or six separate experiments. Data is analysed using a paired Student's T-test by comparing with the control values.

A. Down-regulation of α6 expression



B. Recovery of α6 expression

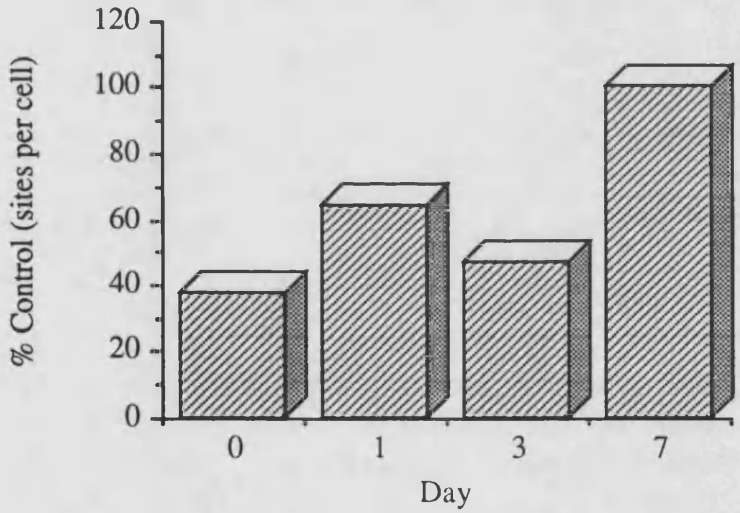


Figure 5.7 Time-course for downregulation and recovery of ZR-75 cell α6 expression

ZR-75 cells were either used directly or cultured in phenol red-free RPMI supplemented with 10% (v/v) CS-FCS in the presence or absence of 17β-oestradiol. Cells were analysed at 1, 3 and 7 days for α6 expression by incubating with a saturating concentration of the mAb or control IgG diluted to the same concentration, followed by incubation with an anti-IgG FITC-conjugate (A). Alternatively, cells were either maintained in medium containing 10^{-9} M 17β-oestradiol or were oestrogen-depleted for 7 days then transferred to oestrogen-supplemented medium for a further 1, 3 or 7 days and α6 expression analysed as described above (B). Cells were examined by FACS analysis. Data was obtained as mean fluorescence intensity which was converted to sites per cell. Antibody dilutions was performed in triplicate and data obtained was combined to give an average value. Results are expressed as % control, ie. integrin expression of ZR-75 cells cultured in the presence of oestrogen. Each histogram represents a single experiment.

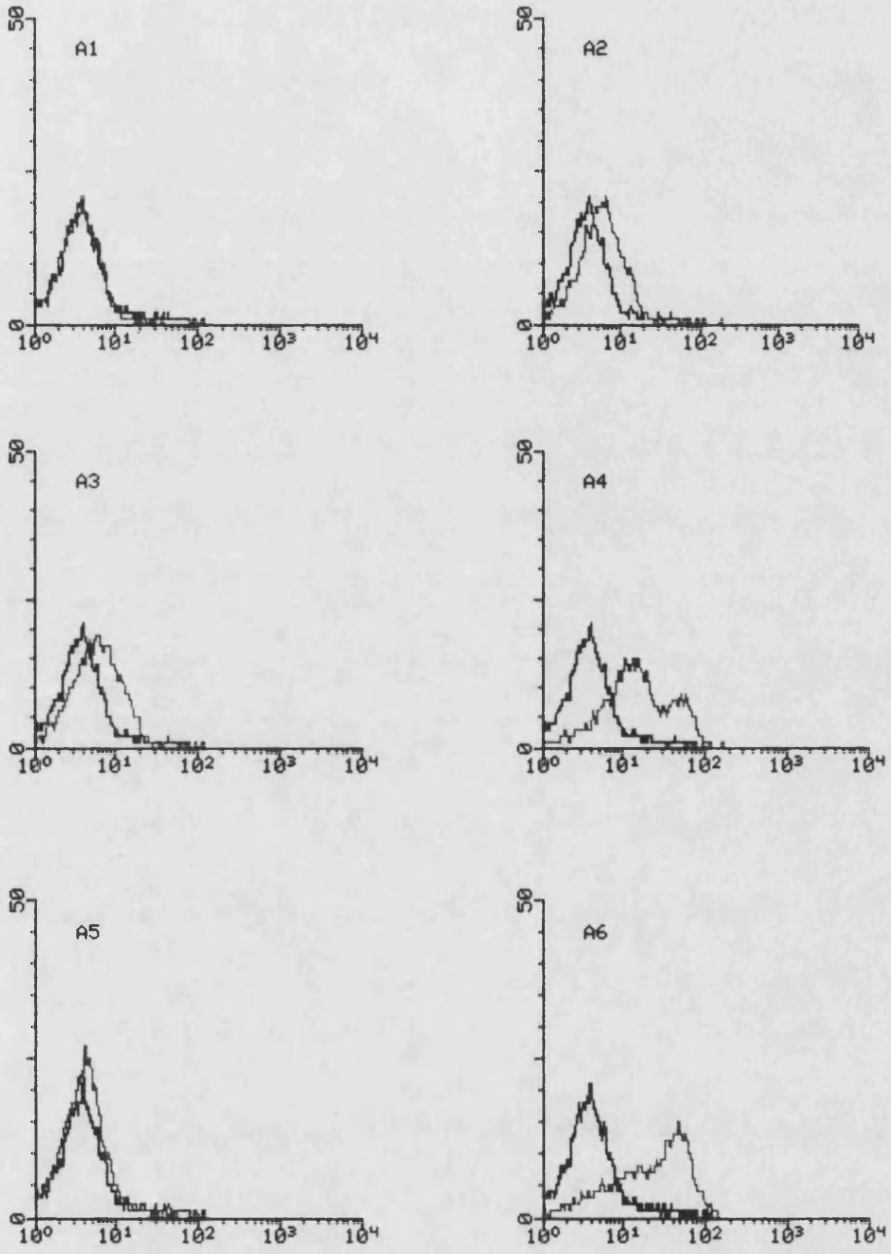


Figure 5.8 Continued...

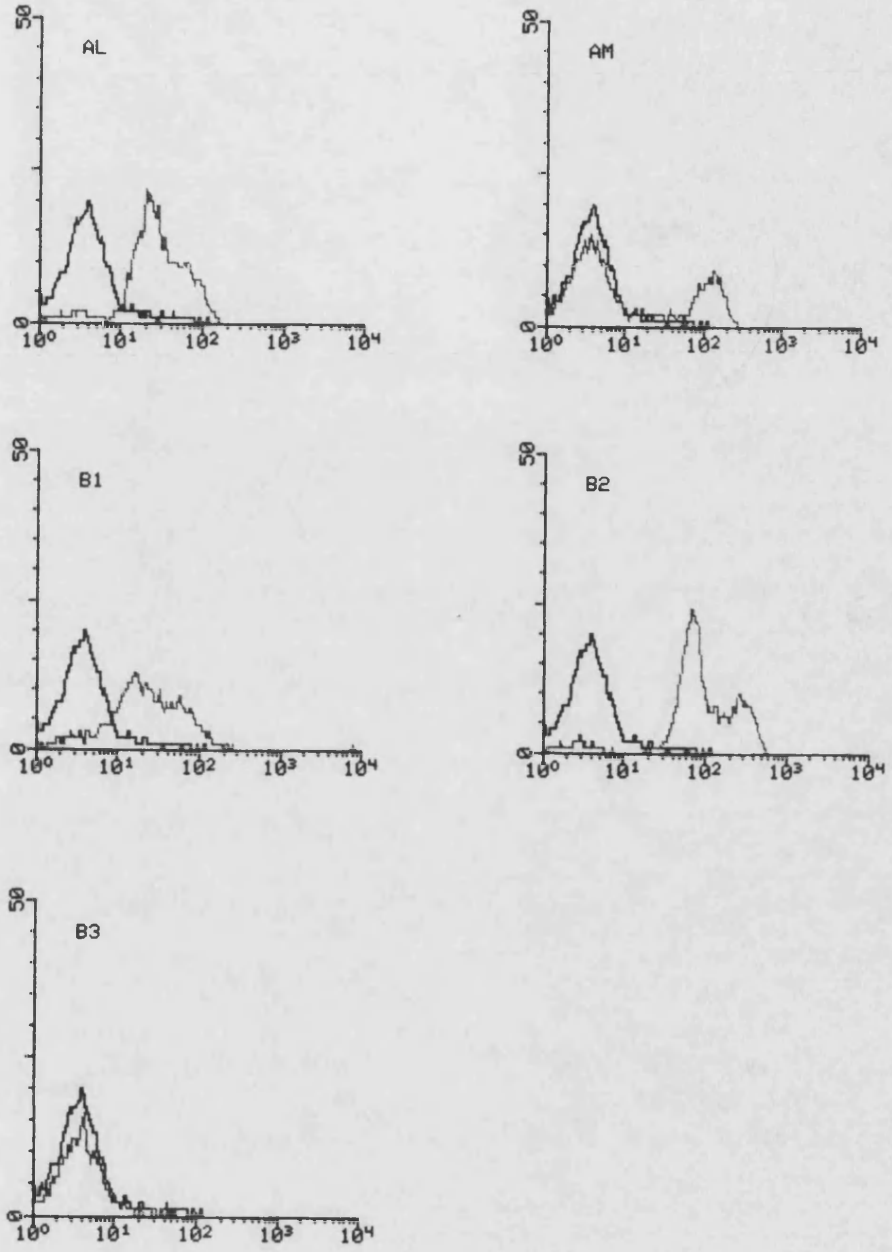


Figure 5.8 FACS analysis of PBMC integrin subunit expression

PBMC were obtained from a normal, healthy female volunteer and incubated with mAbs directed against integrin subunits, all of which were diluted 1/50, or with control IgG diluted to the appropriate concentration. Cells were then incubated with an anti-IgG FITC-conjugate and examined by FACS analysis. Results are expressed as histograms of fluorescence intensity.

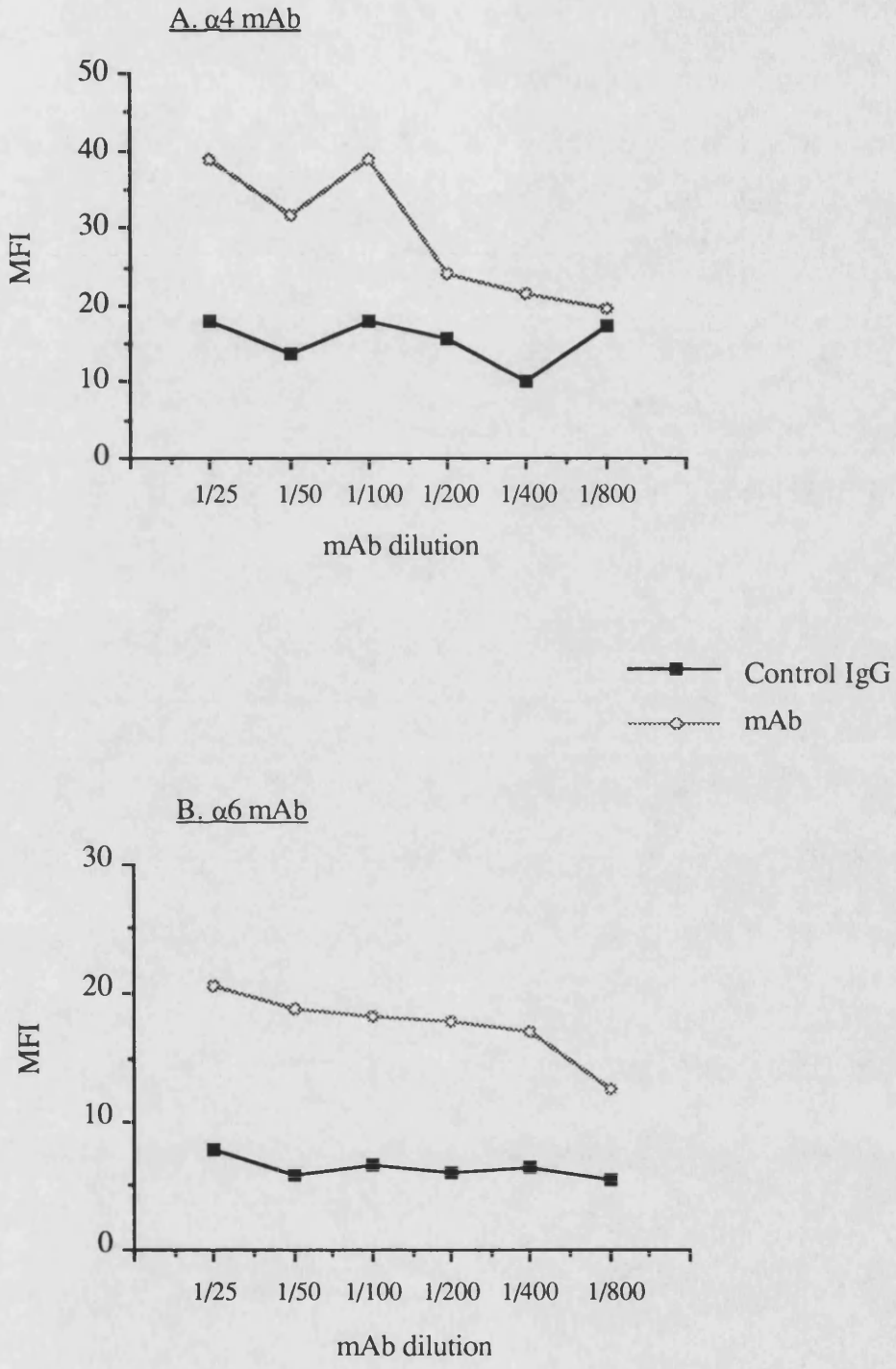
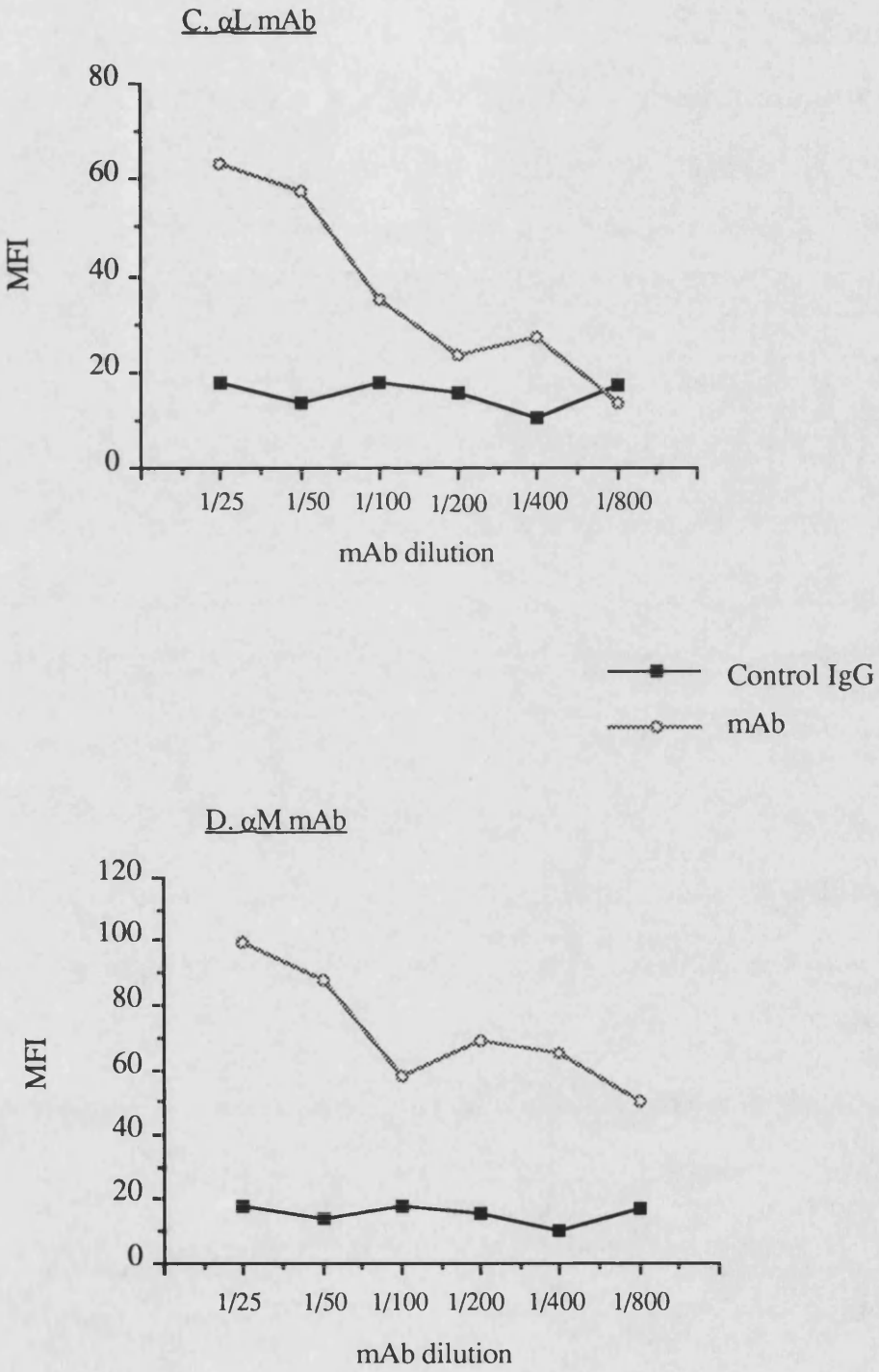


Figure 5.9 Continued...



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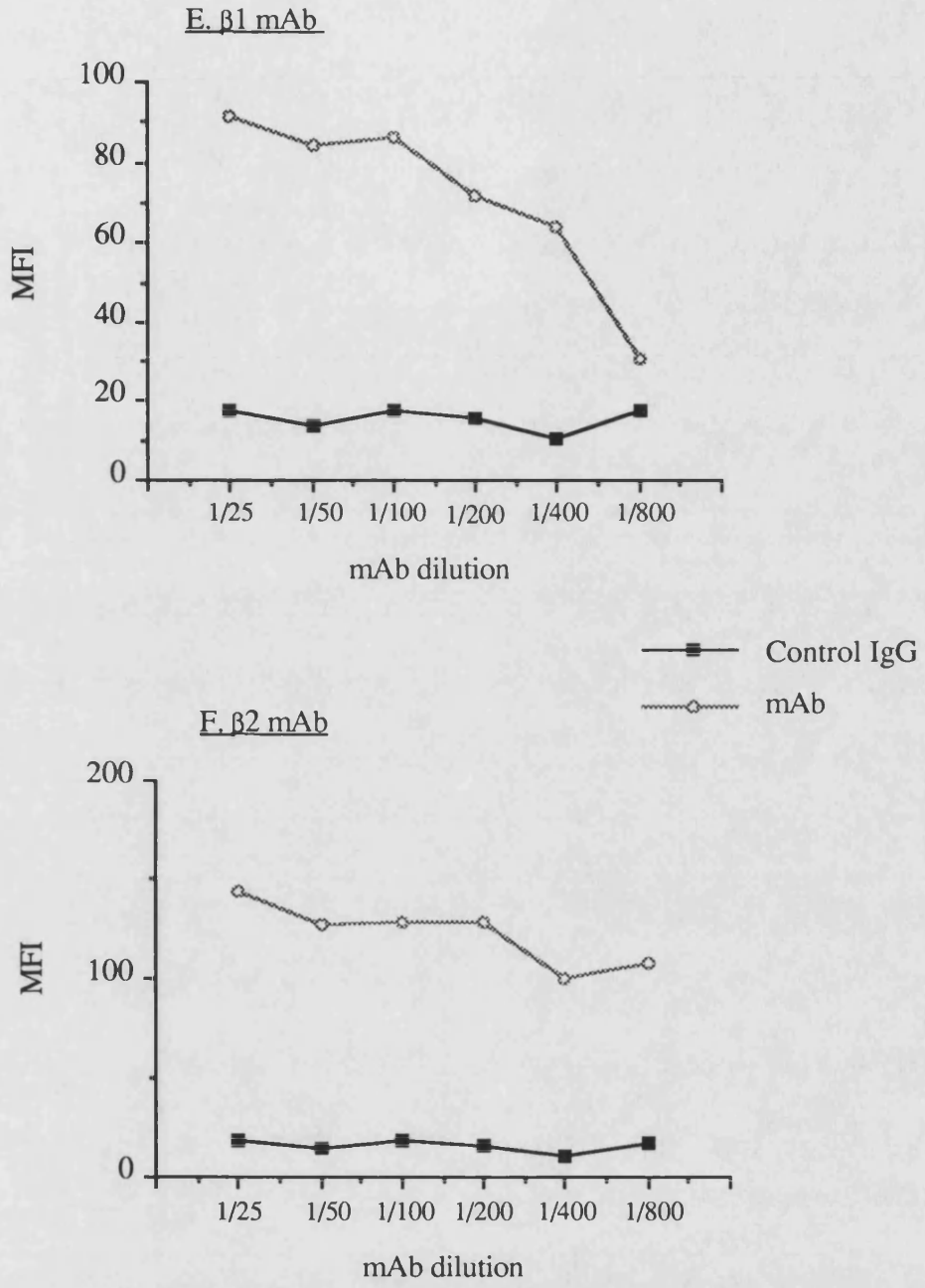


Figure 5.9 Titration of anti-integrin antibody binding to PBMC

PBMC were obtained from a normal, healthy female volunteer and incubated with mAbs directed against the integrin subunits (A) α4, (B) α6, (C) αL, (D) αM, (E) β1 or (F) β2, or with control IgG diluted to the same concentrations. Cells were then incubated with an anti-IgG FITC conjugate and examined by FACS analysis. Results are expressed as mean fluorescence intensity (MFI) and plotted as a titration curve.

A. T cells

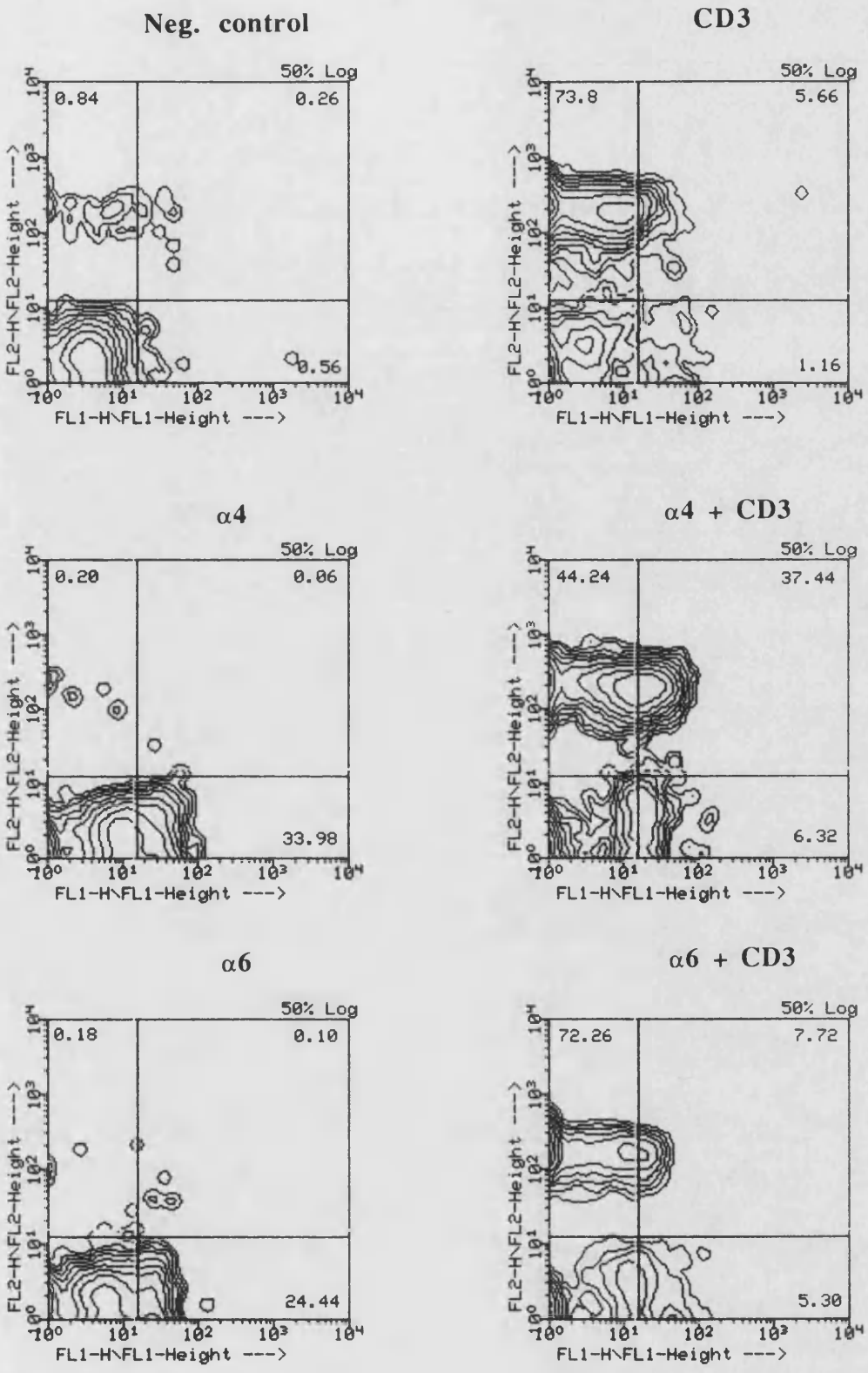
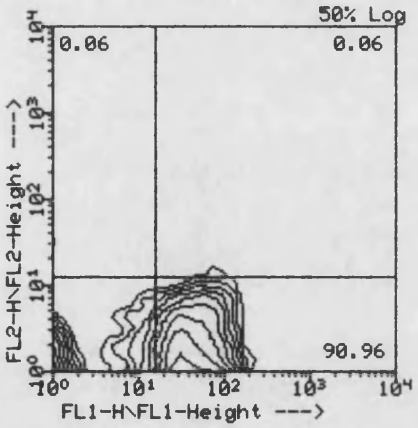
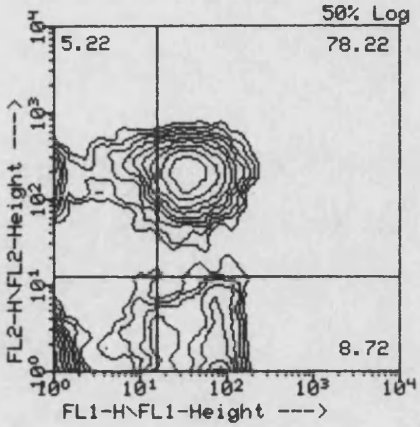


Figure 5.10 Continued...

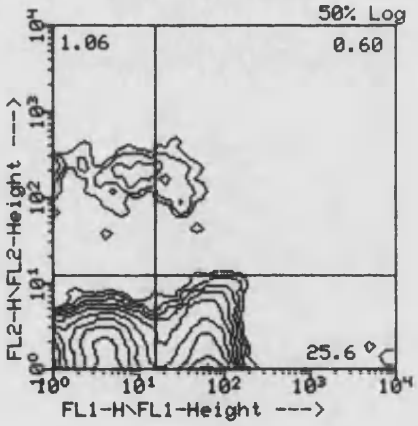
αL



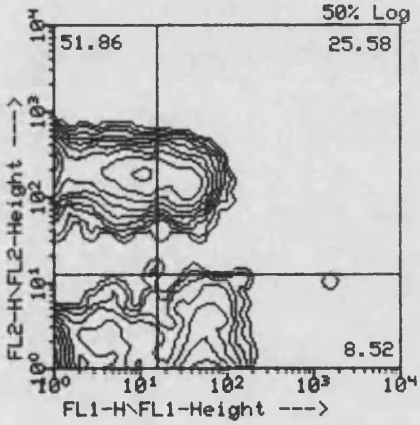
αL + CD3



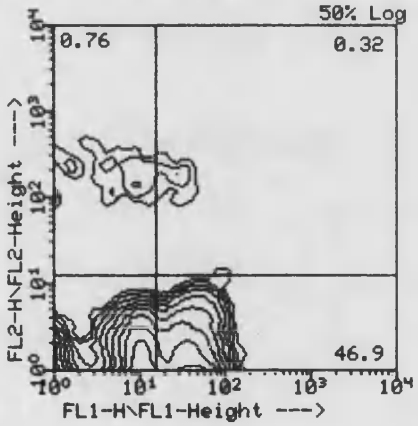
αM



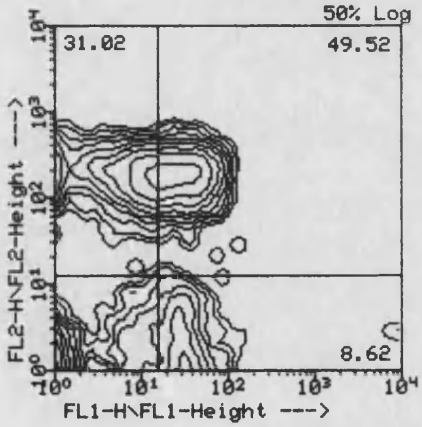
αM + CD3



β1



β1 + CD3



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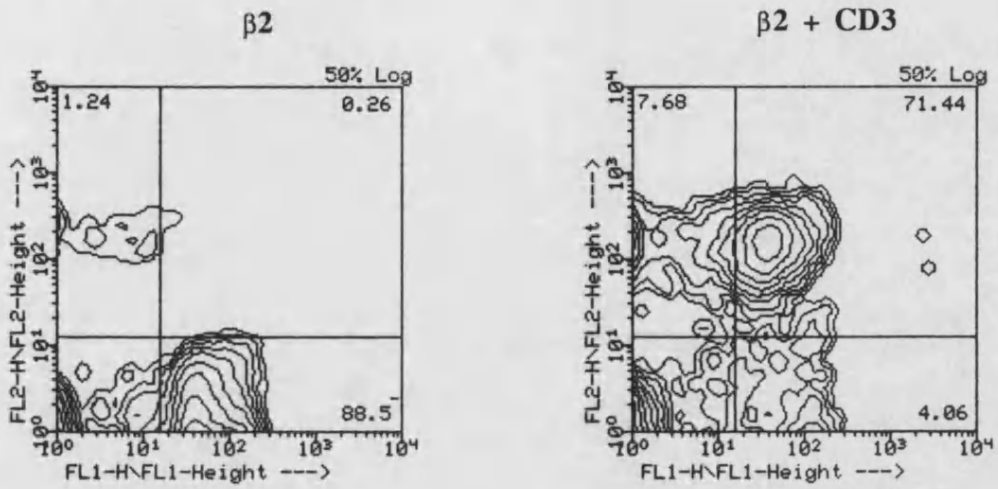


Figure 5.10 Continued...

B. Monocytes

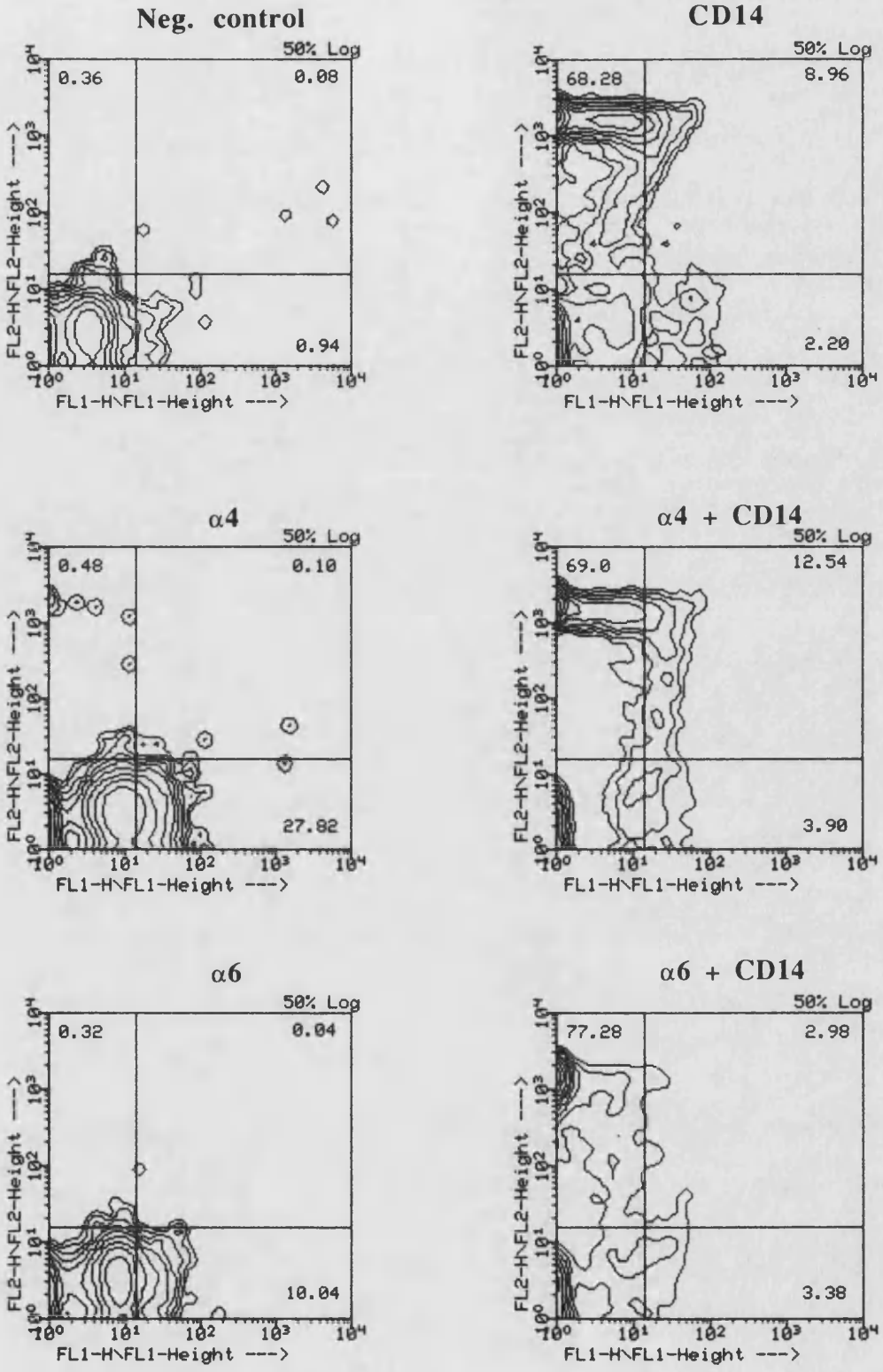
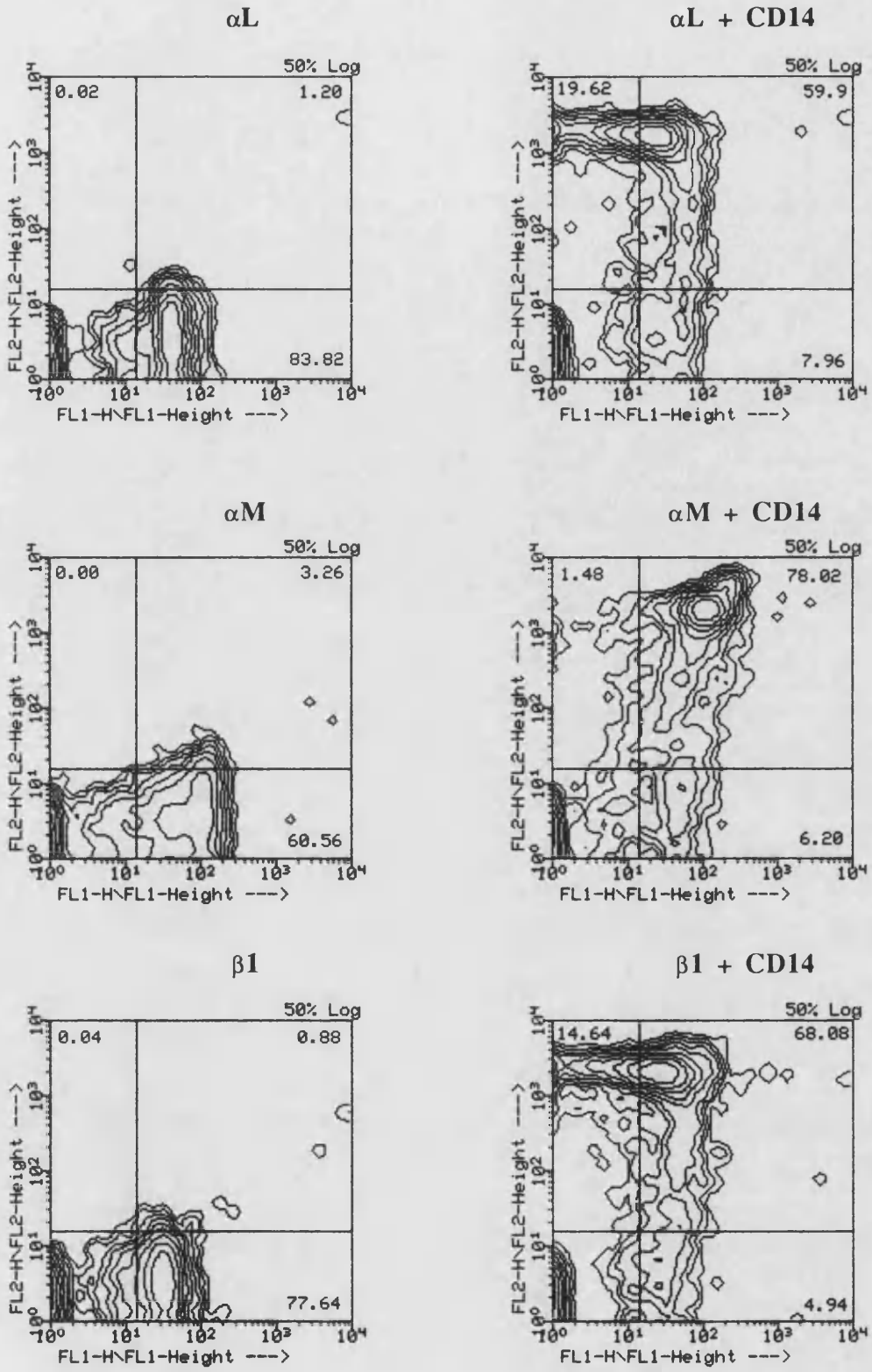


Figure 5.10 Continued...



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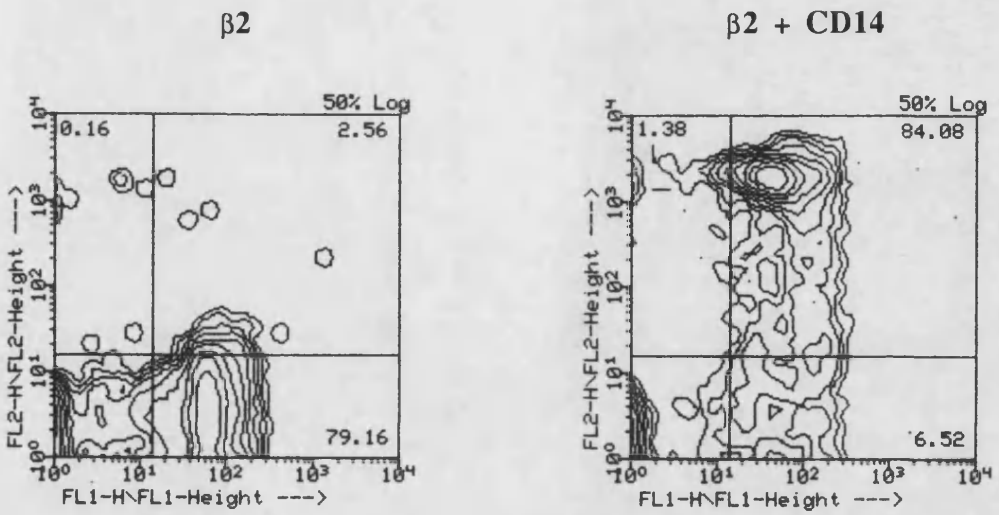

Figure 5.10 PBMC separation and dual labelling for integrin subunit expression

PBMC were obtained from a normal, healthy female volunteer and were further separated into T cells and monocytes. T cells and monocytes were incubated with anti-α4, -α6, -αL, -αM, -β1 or -β2 mAbs and either anti-CD3 (A) or anti-CD14 (B) R-phycoerythrin-conjugated mAbs, respectively. Cells were then incubated with an anti-IgG FITC-conjugate and analysed by FACS analysis. Results are expressed as contour plots of R-phycoerythrin fluorescence versus FITC fluorescence, with the number of cells in each quadrant entered as a percentage of total cells.

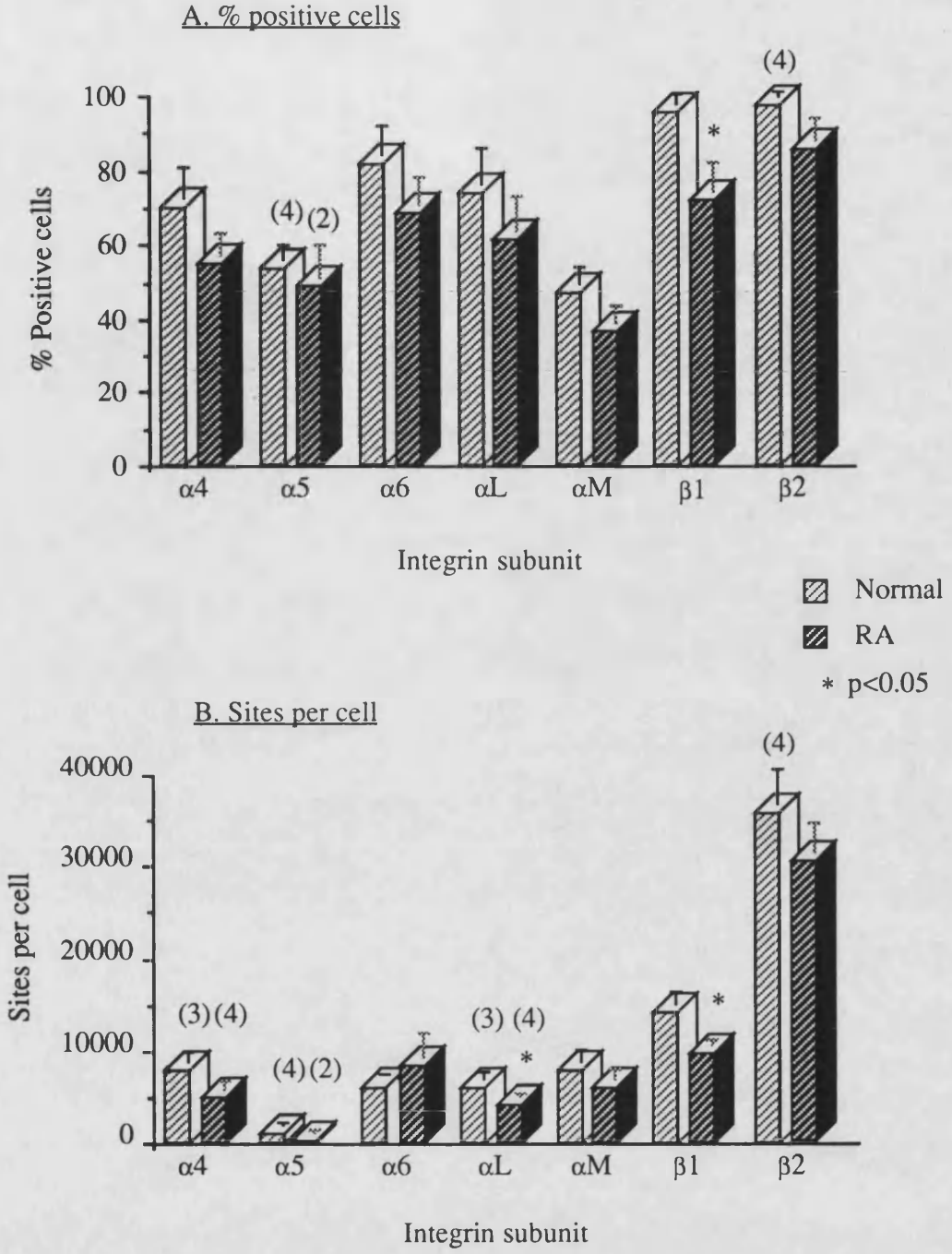

Figure 5.11 Basal integrin subunit expression on control and RA PBMC

PBMC were obtained from one pre-, one peri- and one postmenopausal female and two male RA patients and from age- and sex-matched normal controls. Cells were incubated with anti-α4, -α5, -α6, -αL, -αM, -β1 and -β2 mAbs at saturating concentrations, or control IgG diluted to the same concentration, followed by an anti-IgG FITC conjugate. Cells were then examined by FACS analysis. Data was obtained as mean fluorescence intensity which was converted to sites per cell, or % positive cells relative to the control IgG which was set at 5%. Antibody dilutions was performed in triplicate and data obtained was combined to give an average value. Results from are expressed mean ± SEM % positive cells (A) or sites per cell (B). Each histogram represents five separate experiments, unless otherwise stated. Data is analysed using an unpaired Student's T-test by comparing control and RA values.

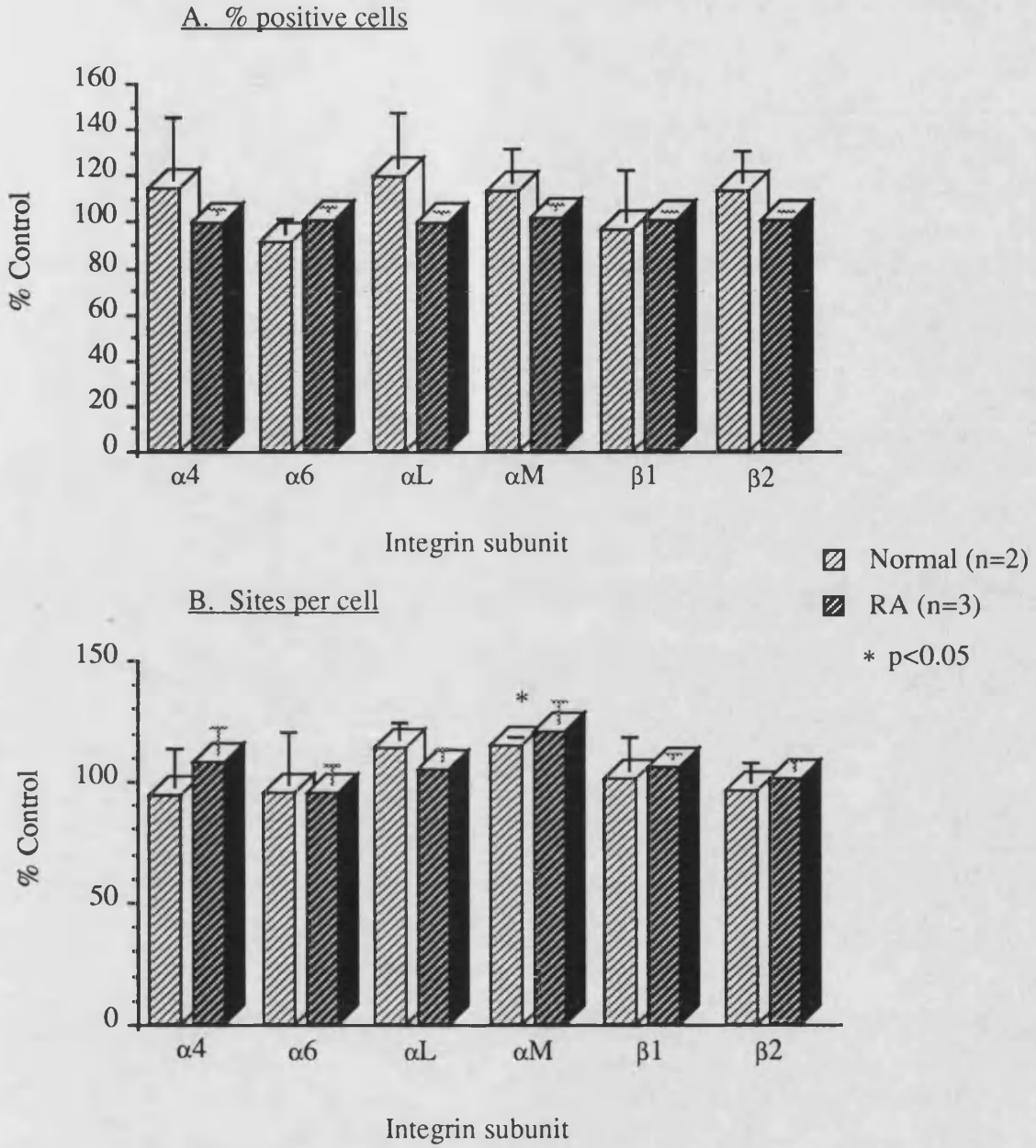

Figure 5.12 Oestrogen modulation of control and RA PBMC integrin subunit expression

PBMC were obtained from one pre-, one peri- and one postmenopausal female and two male RA patients and from age- and sex-matched normal controls and cultured in phenol red-free RPMI supplemented with 10% (v/v) CS-FCS, in the absence or presence of 10^{-9} M 17β-oestradiol. After 48 hours, cells were incubated with anti-α4,-α6, -αL, -αM, -β1 and -β2 mAbs at saturating concentrations, or control IgG diluted to the same concentration, followed by an anti-IgG FITC conjugate. Cells were then examined by FACS analysis. Data was obtained as % positive cells relative to the control IgG which was set at 5% (A), or mean fluorescence intensity which was converted to sites per cell (B). Antibody dilutions were performed in triplicate and data obtained was combined to give an average value. Results are expressed as % control, ie. cells cultured in the absence of oestrogen. Each histogram represents two or three separate experiments. Data is analysed using a paired Student's T-test by comparing to the control values.

CHAPTER SIX

DISCUSSION

There is much evidence to date which supports a role for the sex hormones in immune regulation. Females exhibit an enhanced capacity to mediate both humoral immunity, seen in terms of increased antibody production and resistance to infections, and cell-mediated immunity, in terms of increased skin-graft or tumour rejection capacity and relative resistance to tolerance (reviewed by Grossman, 1984; Lahita, 1990). There is also a female preponderance in many autoimmune conditions, including RA and SLE. Animal data provides additional evidence for sex hormone modulation of the immune system in that the incidence of certain autoimmune diseases is sex-linked and can be altered by gonadectomy and hormone replacement (reviewed by Grossman, 1984; Ansar Ahmed & Talal, 1990).

When considering situations of altered hormonal balance, such as pregnancy and the menopause, different effects on the autoimmune conditions RA and SLE are seen, in that a remission can occur during pregnancy in RA but symptoms are often exacerbated in SLE (see Introduction, Sections 1.3 and 1.4). The onset of RA has been linked to the menopause (McHugh, 1990), whereas susceptibility to SLE is greatest during child-bearing age (reviewed by Lahita, 1985). This has been simplified to sex hormones having differing effects on T cells and B cells, with RA considered a T cell-mediated disease and SLE mediated by B cell hyperactivity. In this hypothetical scheme of events, elevated sex hormone levels, or fluctuations in levels, would potentiate B cell activity and hence trigger or perpetuate SLE. In contrast, loss of the sex hormones, or a sudden fall in levels, modulates T cell functioning in such a way that the onset or worsening of RA occurs. In addition, animal data suggest that oestrogen is beneficial in models of arthritis, whereas this hormone is detrimental in models of SLE and androgens are beneficial. However, the vast array of potential contributory sex hormones, releasing hormones and metabolites, and the complexities of the immune system mean that this hypothesis is likely to be a gross oversimplification of *in vivo* interactions.

When studying the effects of individual hormones in *in vitro* assays of immunity, using mixed cell populations or specific cell types, the results are often contradictory and it is difficult to extrapolate findings to the *in vivo* situation. The immune system would appear to be central to the pathogenesis of RA, therefore any studies of sex hormone effects in this disease are directed to looking at hormonal modulation of immune function. The aim of this project was to investigate the role of sex hormones, in particular oestrogen, on *in vitro* models of immune cell activity, considered to be directly relevant to cellular events occurring in RA.

6.1 SEX HORMONE MODULATION OF CYTOKINE EXPRESSION

In RA, immune cells infiltrate the joint and release factors such as cytokines which have the potential to mediate many of the changes which occur within the joint in this disease. In particular, IL-1 and TNF-α have the capacity to be at least partly responsible for the destruction of the joint architecture, to initiate inflammatory reactions and systemic effects, as well as perpetuating the disease by increasing the production of one another, other cytokines such as IL-6, and the expression of various adhesion molecules (see Introduction, Sections 1.7 and 1.8).

To investigate the effect of 17β-oestradiol and testosterone on PBMC cytokine production, concentrations of 10^{-8}-10^{-12} M were employed, which encompasses the natural range of endogenous hormone levels. Plasma testosterone, ie. free and bound, is approximately $22X10^{-9}$ M and oestrogen levels in women peak at around $2X10^{-9}$ M during the menstrual cycle and reach $1X10^{-7}$ M during pregnancy. Results of studies in Chapter Three failed to demonstrate an effect with either of these sex hormones on the release of IL-6 or TNF from PBMC obtained from normal, healthy controls, although LPS was still able to induce significant release of the two cytokines, illustrating that the cells were responsive in culture. In contrast, Ralston *et al.* (1990) reported a dose-dependent inhibition of PBMC TNF release with concentrations of 17β-oestradiol from 10^{-6} to 10^{-12} M. However, this was only shown for postmenopausal women and no consistent effect was found with either premenopausal females or males, whereas the negative findings reported here were obtained using pre- or perimenopausal female and male controls. Testosterone was found to be inactive in all groups tested. It was also shown that an anti-TNF-α mAb could neutralise the TNF released by the PBMC, indicating that this protein was responsible for the cytotoxic activity measured in the bioassay (Ralston *et al.*, 1990).

The postmenopausal females included in experiments by Ralston's group were suffering from osteoporosis. In their *ex vivo* studies, Pacifici *et al.* (1989) demonstrated that IL-1 release by circulating monocytes from osteoporotic postmenopausal females was significantly reduced following ovarian steroid therapy. Levels of IL-1 were constitutively high in this group and it is possible that oestrogen is only active where such an abnormal production of IL-1 occurs (Stock *et al.*, 1989). Nevertheless, there have also been reports that 17β-oestradiol can modulate IL-1 release from cultured blood cells where there is no underlying abnormality. Using peripheral blood monocytes Polan *et al.* (1988) found that low concentrations (10^{-9}-10^{-10} M) of 17β-oestradiol were stimulatory and high concentrations (10^{-7} M) inhibitory.

As stated, both IL-1 and TNF can induce IL-6 release, and it has been suggested that IL-6 may mediate or amplify the effects of the former two cytokines. In bone cells, physiological concentrations of both 17β-oestradiol (Girasole *et al.*, 1992; Rifas *et al.*, 1992) and testosterone (Girasole *et al.*, 1992) inhibited IL-1- and TNF-induced IL-6 secretion. However, no effect was seen in terms of basal IL-6 production with PBMC. It is possible that the sex hormones are only active in inhibiting cytokine-induced IL-6 production in PBMC, in the same way that bone cell induced IL-6 release was suppressed and only abnormally high IL-1 levels appeared to be affected. However, this possibility has yet to be explored.

The measurement of cytokine expression by bioassay is fraught with difficulties. For example, the bioassay is unlikely to be entirely specific for the cytokine of interest and may be influenced by others present. In addition, cell supernatants contain inhibitory factors which neutralise cytokine activity, hence the supernatant samples have to be diluted considerably before any activity can be measured. Loss of biological activity through long-term storage of samples, or repeated freeze-thawing is also likely to bias results. Because of these problems, mRNA levels were studied as a more accurate predictor of cytokine expression. However, mRNA transcripts are not always translated to protein, and therefore changes in expression at the transcriptional level are not necessarily reflective of post-transcriptional modifications.

The initial results suggested that 10^{-9} M 17β-oestradiol may upregulate TGF-β mRNA expression in control PBMC. This effect was only demonstrated in the absence of serum, as serum contains growth factors, including TGF-β, which are known to increase TGF-β expression and hence mask any additional upregulatory hormonal effect. However, these data could not be normalised, and subsequent experiments which were carried out showed the same pattern of expression for the cytokine and the house-keeping gene, β-actin, illustrating that any variation in expression seen was due to loading inaccuracies rather than an effect of oestrogen *per se*. Filters were probed for mRNA encoding IL-1β, TNF-α, IL-6 and prolactin as well as TGF-β, and both control and RA PBMC were included, using pre-, peri- and postmenopausal females and males, with no difference in cytokine expression seen.

Polan *et al.* (1989) demonstrated that 17β-oestradiol increased IL-1β mRNA levels in LPS-stimulated human peripheral blood monocytes to a maximum at a concentration of 10^{-9} M. At concentrations above this a reduction was seen. The effect of oestrogen on LPS-induced cytokine mRNA expression was not examined here. Treatment of PBMC with 1 μg/ml LPS for 6 hours increased TNF-α and IL-6 mRNA expression, but no

effect was seen for IL-1β. The reason for this is unclear, but may have been due to an inadequate concentration of LPS being employed, or too long a period of incubation, as the effect is transient. Alternatively, the presence of TGF-β1 in the cell cultures may have inhibited any LPS-induced effect on IL-1 mRNA expression, as both IL-1 and TNF have been reported to be inhibited in the presence of LPS and TGF-β1 in human PBMC (Turner *et al.*, 1990). However, TNF mRNA expression was enhanced in the presence of LPS where IL-1 levels remained unchanged, therefore this is unlikely to explain the findings here. Polan *et al.* (1989) utilised 10 μg/ml LPS and measured a maximal stimulation at 3 hours, although with this concentration of LPS the effect as still evident at 24 hours. However, 10 μg/ml is an excessive dose of LPS to employ, and such a high concentration could modulate cell function non-specifically. In such an artificial situation one cannot draw any conclusions from the effects of 17β-oestradiol reported.

In human osteoblast-like cells 17β-oestradiol has been shown to increase TGF-β mRNA expression (Oursler *et al.*, 1991), although this was not always seen with lower concentrations, and it has been suggested that the effect of oestrogen on these cells is at the post-transcriptional level (Keeting *et al.*, 1989). In contrast, Girasole *et al.* (1992) found an inhibition of IL-1- and TNF-α-induced IL-6 mRNA expression with 17β-oestradiol using rodent and human osteoblastic cells and bone marrow-derived stromal cells. This could reflect an inhibition of the IL-6 promoter activity (Pottratz *et al.*, 1992). There are no reports of such an effect with oestrogen on peripheral blood cell TGF-β or IL-6 expression, at the mRNA or protein level.

Prolactin has been reported to be involved in the control of immune function and also in autoimmunity (reviewed by Jara *et al.*, 1991), and cells of the immune system both express prolactin receptors (Russell *et al.*, 1984a) and produce a prolactin-like substance (Montgomery *et al.*, 1987). Oestrogens are important physiological regulators of prolactin secretion, thought to be via a direct effect on the pituitary in the case of 17β-oestradiol (Franks, 1983). Treatment of rat anterior pituitary lobes with oestrogen *in vivo* caused a three- to four-fold increase in prolactin mRNA expression (Martinez-Campos *et al.*, 1991). Therefore, filters were hybridised to a prolactin cDNA probe to investigate the effect of oestrogen on the expression of this hormone in immune cells. Results showed that PBMC express prolactin mRNA, confirming the results of Hiestand *et al.* (1986) demonstrating that rat lymphocyte RNA hybridised to a prolactin-specific probe. However, as stated, no effect was seen with 17β-oestradiol on control or RA PBMC prolactin mRNA expression.

The observation that the pattern of cytokine expression following oestrogen treatment was consistently the same as that for the house-keeping gene β-actin, posed the question was β-actin itself being modulated by this sex hormone. Indeed, it has been reported that the β- and γ-cytoskeletal actins as well as the smooth muscle actin could be induced in rat uterus by treatment with oestradiol (Hsu & Frankel, 1987), although this was only a transient induction, up to two-fold, and the effect was measured *in vivo*. Nevertheless, to ensure that oestrogen modulation of β-actin was not occurring in experiments performed with PBMC, filters were hybridised to a GAPDH cDNA probe. GAPDH has been shown to be transcribed at the same rate in all tissues and therefore regulated post-transcriptionally (Piechaczyk *et al.*, 1984). Results showed the same pattern of mRNA expression for GAPDH as for β-actin and all cytokines examined, thus it was concluded that there was no effect of oestrogen throughout. LPS-stimulation of the cells demonstrated that culturing in serum-free medium did not affect cell activity, and in addition, cells were shown to respond normally to ConA under these culture conditions (results not shown). Therefore it can be concluded that oestrogen has no effect on the expression of any of the cytokines tested here, at the transcriptional level, in circulating mononuclear cells.

The anterior pituitary hormone, FSH, controls oestrogen secretion, and oestrogen in turn can regulate the level of FSH via feedback pathways. It was hypothesised that effects thought to be due to oestrogen may in fact be due to FSH. For example, elevations measured in oestrogen could be a result of a rise in FSH, and the administration of oestrogen may be acting indirectly through modulation of this second hormone. Therefore, the effect of FSH was studied on control PBMC cytokine mRNA expression. The results obtained however were the same as those for oestrogen, with no effect on any of the cytokines tested. At a higher level still, LHRH from the hypothalamus is responsible for controlling the pituitary secretion of both LH and FSH, which in turn regulate sex hormone levels *in vivo*. Rat lymphocytes have been reported to contain immunoreactive LHRH which appeared to be bioactive in that it could induce the secretion of LH from rat anterior pituitary cells (Emanuele *et al.*, 1990), and it has been hypothesised that this may be involved in lymphocyte activation and general immune function (Morale *et al.*, 1991), for example by increasing IL-2 receptor expression (Batticane *et al.*, 1991). The role of this hormone in assays of human PBMC cytokine expression has not been studied but it may prove to be important in the sex hormone/immune system story in the future.

In addition to studying PBMC cytokine expression in the presence of sex hormones, PBMC proliferation was examined as another parameter of immune cell function.

Previous results showed that the proliferative response to PHA was reduced in RA patients compared to healthy controls, whereas the response to an anti-CD3 mAb was more variable, in that proliferation was increased in patients with mild or moderate disease and depressed in patients with severe disease (van den Brink *et al.*, 1992). The results demonstrated here showed little difference between the controls and RA patients in terms of their response to PHA, but the response to anti-CD3 mAb was reduced in several patients, although the overall mean response was not significantly different to that of the controls. It has been reported that T cell responses are dampened down in RA, with T cell-derived cytokines being undetectable at the protein level in the synovial fluid and synovium (reviewed by Brennan *et al.*, 1991), reduced IL-2 production in response to PHA (Combe *et al.*, 1985) and depressed responses in cell-mediated immune reactions (Emery *et al.*, 1984). The latter abnormality could be reversed by the addition of IL-2 (Emery *et al.*, 1984). In these experiments IL-2 was able to potentiate the response to anti-CD3 mAb in certain RA patients, but in others the response to anti-CD3 mAb and IL-2 was as low as with anti-CD3 mAb alone. These responses were thought to correlate with disease activity as there was no correlation between proliferation and sex or age. In the experiments reported in this thesis, neither oestrogen or testosterone had any effect on the response to any of the stimuli tested. van den Brink *et al.* (1992) also failed to demonstrate an effect with these two hormones.

FSH and LH have been found to increase the proliferation of peripheral blood lymphocytes (Costa *et al.*, 1990). The effect was greater in elderly (59-90 years) than young (30-40 years) females, and there was a negative correlation between the effect of FSH and the plasma level of 17β-oestradiol alone, or both 17β-oestradiol and progesterone, in the younger subjects. Preliminary data using PBMC from premenopausal female controls failed to demonstrate any effect of FSH, at comparable concentrations to those used by Costa *et al.* (1990) (results not shown). According to the previous findings, this may relate to higher plasma oestrogen levels.

6.2 OESTROGEN RECEPTOR

The negative results obtained, in terms of an effect of oestrogen on cytokine production or proliferation by PBMC, were supported by further investigation into the activity of the 17β-oestradiol utilised and the presence of ER on PBMC. Cell lines which were reported to be either ER-positive and hence oestrogen-responsive (ZR-75 and T-47D cells), or ER-negative and therefore unresponsive to oestrogen (Hs578T cells), were

employed as a means of studying the efficacy of the batch of oestrogen used for previous experiments and also as positive and negative controls in studies of ER expression.

ZR-75 cells were shown to proliferate in response to 17β-oestradiol, demonstrating that the negative results obtained were not due to an inactivity of the oestrogen employed. However, the reportedly ER-positive cell line, T-47D, was unresponsive, with results identical to those seen with the ER-negative cell line, Hs578T. Details of an ER/PR-negative T-47D clone, obtained by maintaining the cells for one year in oestrogen-free medium, have been described (Murphy *et al.*, 1990). This clone was refractory to both oestrogen and anti-oestrogen treatment,whereas another clone has been described which is oestrogen-insensitive but still retains ER expression (Dabre & Daly, 1990). Therefore it is possible that the T-47D cells used in these experiments, which were present in-house, may have been subjected to culture conditions previously which resulted in the loss of oestrogen responsiveness. As will be described, the cells were shown to express ER mRNA, although ER protein was not detectable.

The response of the ZR-75 cell line to 17β-oestradiol was inhibited by the anti-oestrogen, tamoxifen, illustrating that this was a specific oestrogen effect. When 10^{-9} M 17β-oestradiol was used to induce proliferation tamoxifen had a purely antagonistic effect. However, when a concentration of 10^{-10} M was employed, only concentrations of tamoxifen above 10^{-7} M were inhibitory, whereas lower concentrations potentiated the oestrogen effect. Tamoxifen is known to behave as a partial agonist which would explain this finding.

The ability of a cell to respond to a given ligand is governed by the degree of expression of the specific receptor. Receptors for the sex hormones are commonly present in very low amounts, although in some cells as few as 10000-80000 ER sites per cell is sufficient to mediate a response (Katzenellenbogen *et al.*, 1987). By constructing transfectants expressing varying levels of ER, Webb *et al.* (1992) concluded that below 500000 sites per cell the response was limited, in terms of the number of EREs bound by ER, whereas at higher ER titres spare receptors existed and 'squelching' occurred, whereby the availability of a molecule essential for receptor functioning became limited. These findings demonstrated that under normal conditions there is relatively little occupation of EREs, suggesting that occupancy of responsive elements is not the only parameter controlling the cellular capacity to mediate an oestrogen response.

Previous studies into the presence of ER in the immune system have been carried out using radiolabelled ligand-binding assays. Binding assays require the presence of unoccupied receptor, therefore the low levels of ER reported may be partly due to the inability to detect ER with bound oestrogen, or to the presence of other steroid binding factors, such as SHBG, which would also cause an underestimation of the actual amount of receptor present. A more direct method of measuring receptor expression is by use of specific mAbs. Using the H222 mAb, directed against the ER, in an immunocytochemical assay system, ER protein was detected in ZR-75 cells but not in T-47D cells, Hs578T cells, PBMC, synovial fibroblasts or rheumatoid synovial tissue. ZR-75 cells maintained under normal culture conditions demonstrated a similar degree of ER expression to that achieved with the MCF-7 cell line which was used to prepare control slides for use with the detection kit. The nuclear staining seen was unevenly distributed across the cell population. When ZR-75 cells were cultured in the absence of oestrogen, ER expression could not be demonstrated. One possible explanation for this is that ligand-binding is required for tight nuclear association of the receptor prior to detecting with anti-ER mAbs. Therefore, the failure to detect ER in this situation is not necessarily indicative of a functional downregulation in receptor expression, although a reduction in ER under conditions of oestrogen-deprivation cannot be totally excluded.

The failure of the H222 mAb to detect ER on any cells except known ER-positive breast carcinoma cell lines reflects the limitation of immunocytochemical analysis where receptor levels are constitutively low. The ER-D5 mAb was employed as an alternative, as it has been reported to be a far more sensitive indicator of ER expression. This antibody was raised against a 29 kD cytoplasmic antigen (p29), reported to be related to the ER (Coffer *et al.*, 1985a) and a strong predictor of ER expression (Coffer *et al.*, 1985b). However, the relevance of the antigen to ER has yet to be confirmed. ER-D5-binding was demonstrated for all cells and synovial sections tested. ZR-75 cells gave the strongest cytoplasmic staining, which is in accordance with the ability of H222 mAb to detect ER protein in these cells alone. Synovial fibroblasts were also found to express considerable p29, whereas the staining for the purportedly ER-negative cell line, Hs578T, and for PBMC, was weak and diffuse. The level of p29 expression by cells was also examined by FACS analysis which produced comparable results to those seen histologically.

The p29 antigen has been reported to be present at approximately one thousand times the concentration of ER in oestrogen-responsive tissues. Results with the ER-D5 mAb in breast tumours have shown that all ER-rich samples stain strongly, and those with low or undetectable ER using standard detection methods also showed a certain degree

of positivity with ER-D5 (Coffer *et al.*, 1985b; Cano *et al.*, 1986). Thus, the results with the Hs578T cell line are likely to be indicative of ER expression below the level of detection, and hence functionality. ER mRNA could not be detected in this cell line which further supports this conclusion. The level of p29 expression was also shown to be low in PBMC, although staining patterns using the APAAP procedure were more intense for PBMC obtained from a postmenopausal female RA patient than for a premenopausal female control. Higher levels of p29 have been recorded for breast cancer patients above the age of 50 than those under 50 years (Coffer *et al.*, 1985b). Therefore the results obtained here may reflect the age of the subject, or menopausal status. Alternatively, the increase in p29 expression could be as a result of the RA. However, when a range of RA patients and age- and sex-matched controls were studied for ER-D5-binding by FACS analysis, binding sites were generally lower in the RA patients than the controls, although the difference was not significant. When grouping into age there was also no significant difference in p29 expression. FACS analysis is a qualitative and quantitative method of assessing the degree of expression of both cell surface and intracellular markers, whereas APAAP staining is largely qualitative. In addition, there is greater scope for inter-experimental error with the latter technique.

The low expression of p29 antigen in the ER-negative cell line, Hs578T, and the comparably low levels of p29 in PBMC, questions the previous reports of ER in immune cells, and, if present, its functionality. Cohen *et al.* (1983) were the first group to publish data which suggested that ER were expressed exclusively by the CD8+ T cell population. This was supported by Stimson in 1988 who also reported the CD8+ subset to be positive for ER, and the CD4+ cells negative. Therefore the failure to detect ER in the mixed PBMC population could be due to the low numbers of CD8+ T cells present. Additionally, if p29 antigen expression is also restricted to CD8+ T cells, this would also explain the minimal staining seen with ER-D5 mAb. However, monocytes have also been reported to possess ER (Gulshan *et al.*, 1990), which would suggest that an imbalance of ER-negative and ER-positive cells within the PBMC population is not the reason for the lack of detection of the receptor. In theory the lower ER-D5 binding in RA PBMC may reflect an underlying deficiency in the ER-expressing cell population. A reduction in CD8+ cell numbers has been reported in RA (Veys *et al.*, 1982). However, this is controversial, and if the CD8+ cell subset is depleted in the RA patients included in the experiments reported in Chapter Four, the levels are unlikely to be reduced to such an extent that p29 expression would be affected. In addition, if monocytes do indeed express ER, one would expect p29 antigen to be expressed simultaneously, which would likely counteract any underlying CD8+ cell deficiency in terms of ER-D5 binding.

Synovial tissue from a postmenopausal female RA patient was observed following incubation with ER-D5 mAb. Distinct areas of positive staining were seen and closer examination revealed the most intense staining to be localised to clusters of infiltrating cells. The majority of infiltrating cells were negative which suggests that the predominant CD4+ T cells occurring here do not express p29. Therefore, the cells which appeared to express significant levels of p29 antigen could be CD8+ T cells, or alternatively B cells or macrophages. To date there are no reports of ER on B cells, although there have been reports of oestrogen effects, in particular on the CD5+ subset (see Introduction, Section 1.6.3). If macrophages are responsible for the intense cell staining within the clusters, stronger staining might have been expected in the synovial lining layer. However, only weak, diffuse staining was observed in this area. Alternatively, the macrophages present within the cell clusters may be activated in some way, perhaps by other inflammatory cells present, which results in an upregulation of p29 expression. The weak staining of the lining layer was somewhat unexpected following the strong ER-D5 binding seen with synovial fibroblasts, although it is difficult to extrapolate from the results with a single cell type maintained in culture to those with a network of cells in an *ex vivo* situation. Further investigation by dual-labelling sections with the ER-D5 mAb and mAbs directed against different cell markers, including CD8, is required before any firm conclusion can be made regarding the phenotype of cells expressing p29 antigen.

In a recent publication, Cutolo *et al.* (1993) also used the ER-D5 mAb to detect ER in rheumatoid synovium, and performed dual-labelling to assess its cellular distribution. They demonstrated p29 expression in macrophage-like synoviocytes and CD8+, CD29+ T cells, which supports the previous findings of ER in CD8+ cells in peripheral blood (Cohen *et al.*, 1983; Stimson, 1988) and monocyte/macrophages (Gulshan *et al.*, 1990), and provides further evidence for a direct correlation between nuclear ER protein and cytoplasmic p29. The location of p29 antigen, and presumably therefore ER, within RA synovium, and in particular its association with cells capable of contributing to the disease process, suggests that oestrogen may be of importance in the joint milieu.

The progression from studies of ER at the protein level to investigations of its transcriptional expression were necessitated by the failure to detect ER protein *per se* in PBMC using specific mAbs. Northern blot analysis of ZR-75 cell total RNA using a specific human ER cDNA probe appeared to demonstrate the expected 6.2 kb transcript, but neither T-47D cell, Hs578T cell or PBMC RNA hybridised to the ER cDNA. Reverse transcription of total RNA and amplification of the cDNA product

using Taq polymerase and specific oligonucleotide primers, designed to frame a region of the ER ligand-binding domain, provided a more sensitive method for the detection of ER mRNA. RNA from both the ZR-75 and T-47D breast carcinoma cell lines amplified to give the correct sized product. Sequencing of the PCR product for ZR-75 cells confirmed this to be identical to the oestrogen-binding domain of the human ER cDNA (Greene *et al.*, 1986). Hs578T cells failed to demonstrate a PCR product, in support of this being an ER-negative cell line (Hackett *et al.*, 1977). The expected DNA fragment was also obtained from synovial fibroblasts, which agrees with the relatively strong binding of ER-D5 mAb in these cells.

Reverse transcription of PBMC RNA and amplification by PCR with the ER-specific oligonucleotide primers resulted in a clear DNA product, illustrating that PBMC possess ER mRNA. The expression of ER mRNA was demonstrated consistently for female controls using this method, whereas in two of the three male control PBMC RNA samples the PCR product was present in only low amounts or was undetectable. The male control demonstrating the greatest level of ER mRNA expression, in terms of the amount of PCR product observed, was receiving steroid therapy which may have upregulated ER levels transcriptionally. Interestingly, PBMC obtained from two premenopausal female controls demonstrated a slower migrating species, in addition to the predicted fragment. This appeared to be between 320 and 350 bp, compared to the expected 287 bp product. It was initially considered that this may be related to endogenous hormone levels as it appeared to be limited to a subpopulation of the female controls and was not demonstrated with RNA from male donors. However, when the Taq polymerase was obtained from a different source, and a $MgCl_2$ titration was carried out using ZR-75 cell cDNA, the same two bands were seen at concentrations of $MgCl_2$ above 1.5 mM. Hence, despite the fact that the concentration of $MgCl_2$ used previously was 1.5 mM and that the two bands were only detected in certain RT-PCR reactions, it was concluded that the second PCR product was a PCR artefact. This was further substantiated when a band-stab method was employed to attempt to separate the two PCR products (Bjourson & Cooper, 1992) and a single PCR product was consistently recovered on re-amplification, indicating that the second band was an artefact of the original, perhaps due to differential splicing. The inability to separate the two products meant that they could not be sequenced and hence the exact identity of the second PCR fragment could not be verified.

Bellido *et al.* (1993) also used the RT-PCR method to detect ER mRNA and developed a pair of oligonucleotide primers to frame a 350 bp region of the DNA-binding domain of the murine uterine ER for this purpose. Using these primers with reverse transcribed

RNA from two bone marrow-derived stromal cell lines and an osteoblast-like line, as well as from the ER-positive breast carcinoma cell line, MCF-7, the 350 bp fragment was detected as was a second faster migrating product. The second PCR product appeared to be of similar size to that observed with PBMC RNA here, which may suggest that this is a genuine result and not a PCR artefact as originally thought. It is possible that the $MgCl_2$ concentration determined the ability of the primers to bind to a different site, although the fact that primers described here, and those used by Bellido *et al.* (1993), were directed against two separate and both highly conserved domains of the receptor, makes it unlikely that the primers would have bound to regions other than these.

The results of these experiments demonstrate that PBMC express ER mRNA only at extremely low levels, and the translation of this into ER protein has yet to be confirmed, although the expression of the p29 antigen suggests that ER is present at the protein level. The recent development of a mAb directed against the DNA-binding domain of the ER may provide a sufficiently sensitive method to detect ER in PBMC immunohistochemically. The use of this antibody has provided the first convincing evidence of an immunohistochemical location of the ER in osteoblasts and osteoclasts (Mizuno *et al.*, 1992; Ikegami *et al.*, 1993), in situations where the H222 mAb has been unsuccessful (Colston *et al.*, 1989). This could provide a useful tool in terms of allowing relatively small numbers of cells to be analysed for ER expression by flow cytometry, giving an accurate analysis of receptor sites per cell and expression by specific cell populations. Sumner *et al.* (1991) reported that the saponin-permeabilisation technique for the detection of intracellular antigens can be used for dual-labelling with antibodies directed against cell-surface markers added simultaneously, as permeabilisation did not significantly alter antibody binding to the cell surface. This would be useful with anti-ER mAbs and also the ER-D5 mAb.

6.3 SEX HORMONE MODULATION OF INTEGRIN EXPRESSION

The ZR-75 cell line requires oestrogen to maintain anchorage-dependent growth in culture. Culturing the cells in oestrogen-depleted medium resulted in a significant reduction in cell growth and, unexpectedly, a change in morphology. This was observed after only three days of culture under these conditions, and appeared to be maximal after seven days. The change in ZR-75 cell morphology, including reduced cell adherence and colony formation, was associated with downregulation of the integrin subunit, α6. α6 associates with the β1 subunit to form VLA-6, a receptor for

laminin. The other integrin subunits expressed on this cell line, α2, α3 and β1, were not altered following the period of oestrogen deprivation. α2β1 (VLA-2) is a receptor for collagen and laminin and α3β1 (VLA-3) forms a receptor for fibronectin, laminin and collagen.

Binding to laminin in the basement membrane is thought to be important in tumour metastasis, which is a complex process involving interactions between cancer cells and the basement membrane, which forms a natural barrier to be crossed when leaving the primary tumour, in intra- and extravasation and in the penetration of muscles and nerves (Liotta, 1986). Therefore it could be hypothesised that downregulation of α6 expression would result in reduced metastasis due to reduced binding to laminin. A considerable proportion of carcinomas of the breast are oestrogen-dependent and can be controlled therapeutically with the anti-oestrogen, tamoxifen. According to these observations, continued oestrogen-stimulation of ER-positive breast carcinoma tissue would maintain high α6 levels and hence increase the likelihood of tumour metastasis. However, it is known that ER-negative tumours are less well differentiated and more invasive than their ER-positive counterparts and oestrogen-depletion of the ZR-75 cell line appeared to cause a downregulation of ER, in conjunction with the α6 integrin subunit.

The spectrum of integrin subunit expression on ZR-75 cells correlated with that defined for breast carcinoma tissues (D'Ardenne *et al.*, 1991). In normal breast tissues α6 staining was shown to be weak in the epithelial cytoplasm, moderate in cell membrane and most intense in the basement membrane. In contrast, carcinoma tissue demonstrated variable cytoplasmic/cell staining but there was a consistent loss of basement membrane staining for α6, which correlated with a reduction in the ligand, laminin. Gould *et al.* (1990) also demonstrated reduced α6 expression in all breast carcinomas, in conjunction with a reduction in β1 levels. They concluded that the difference in α6 expression between carcinomas may explain their local invasiveness and metastatic behaviour. Oestrogen has been reported to increase the expression of the non-integrin 67 kD laminin receptor, thought to correlate directly with the metastatic phenotype of cancer cells (van den Brule *et al.*, 1992).

It has also been proposed that laminin is not involved in tumour cell migration (Coopman *et al.*, 1991), rather it may be that the loss of cell surface integrin expression is solely responsible for the morphological changes seen. Invasive breast carcinomas are characterised by a progressive loss of tissue organisation, including reduced differentiation, as was observed with the ZR-75 cell line *in vitro*. Pignatelli *et al.* (1992)

reported a reduction in the expression of both α2β1 and α6β4 receptors in invasive carcinomas. Therefore the reduction in α6 expression in ZR-75 cells may correlate with a reduction in the level of the α6β4 receptor, rather than α6β1, as the level of the β1 subunit was unaltered. The expression of the fibronectin receptor subunit, α3, has also been proposed as an important factor in tumour cell metastasis (Gould *et al.*, 1990).

The relevance of the downregulation of α6 expression in ZR-75 cells *in vitro* following a period of culture in oestrogen-depleted medium is only speculative. It would seem contradictory that the removal of oestrogen, considered to be detrimental in breast cancer, leads to the loss of integrin subunit expression, which has been proposed as being directly related to tumour cell differentiation and invasiveness. α6 expression could be recovered after reculturing the cells in the presence of oestrogen. The effect of long-term oestrogen deprivation was not studied and the effects of this on α6 expression at the mRNA level were also not examined. However, oestrogen has many effects, of which integrin expression may prove to be inconsequential. The effects of oestrogen in breast cancer are unlikely to be as defined as originally thought and a recent report demonstrating that, following transfection of ER into an ER-negative cell line, oestradiol could inhibit metastatic activity (Garcia *et al.*, 1992), indicates that factors other than the ER appear to be involved.

Changes in integrin expression have also been reported to occur as a result of the inflammatory process, due to factors such as cytokines released at the inflammatory site, which may be important in conditions such as RA (reviewed by Panayi, 1993). FACS analysis of PBMC obtained from healthy controls demonstrated the expression of α4, α6, αL, αM, β1 and β2 integrin subunits. αLβ2 (LFA-1) and αMβ2 (CR3 or Mac-1) are members of the leukocyte integrin family and are important in immune regulation. α4β1 and α6β1 perform interactions with the extracellular matrix which is also critical for immune surveillance (reviewed by Springer, 1990). Dual-labelling of cells confirmed that T cells express predominantly αL and β2, whereas αM and β2 were present in the highest levels on peripheral blood monocytes. All subunits found on PBMC were also found to be expressed on both T cells and monocytes. The αMβ2 receptor is not found on lymphocytes and therefore the demonstration of this subunit in the T cell population might have been a result of PMNL contamination. The high levels of αM expression found on monocytes could be due to inadvertent activation of the cells during the experimental procedure.

Results of experiments comparing integrin subunit expression on PBMC from controls and RA patients showed a general trend of reduced levels on RA cells. This was

significant for β1 in terms of the percentage of cells expressing the subunit and the number of sites per cell. The reduction in αL subunit was also significant, but only in terms of the percentage of positive cells. McCarthy *et al.* (1992) found a reduction in β1 expression when examining RA peripheral blood leukocytes, and also reported an increase in αM. As the basal αM levels were thought to be modulated by cell preparation procedures, conclusions regarding the expression of this subunit in control and RA PBMC cannot be drawn. The VLA-4, -5 and -6 receptors are expressed on naive T cells, and memory T cells express three- to four-fold higher levels than resting cells (Shimizu *et al.*, 1990). The initial investigation into PBMC integrin expression failed to demonstrate any appreciable anti-α5 mAb binding. However, subsequent studies showed low levels of this subunit. The expression of VLA-4, -5 and -6 was not significantly different on control and RA PBMC, whereas it might have been expected to see increased expression on RA cells as a result of activation *in vivo*. Binding of synovial fluid T cells to fibronectin was increased compared to the binding of peripheral blood T cells in RA, which appeared to be mediated by the VLA-5 (Garcia-Vicuna *et al.*, 1992) and/or VLA-4 (Rodriguez *et al.*, 1992) receptors. It is possible that any modulation of integrin expression in RA occurs exclusively at the inflammatory site, on synovial cells. It has also been found that the expression and function of integrins are not mutually exclusive, in that the increased cell adhesion can occur as a result of the activation of existing molecules, without the necessity for increased expression.

Incubation of control and RA PBMC with oestrogen did not alter basal levels of the various subunits. Cytokines such as IL-1 and TNF can modulate integrin expression (reviewed by Pober & Cotran, 1990) and may be important in enhancing cell adhesion in RA. It is possible that oestrogen may modulate the expression of adhesion molecules which are upregulated as a result of the inflammatory process. In this respect, oestrogen has been shown to potentiate TNF-induced upregulation of ICAM-1 and ELAM-1 on endothelial cells (Cid *et al.*, 1992). ICAM-1 has been reported to be increased in RA synovial fluid macrophages compared with peripheral blood monocytes, and GM-CSF, IFN-γ, TNF-α and IL-1 have the capacity to increase ICAM-1 expression on peripheral blood cells to a level comparable to that on synovial cells (Diaz-Gonzalez & Alvaro-Gracia, 1992). Therefore it would be of relevance to study the effect of oestrogen on ligands as well as receptors.

Integrin expression in rheumatoid synovial tissue was not studied. However, a recent report found that the α6 subunit was the only consistent subunit to be expressed by synovial lining cells, and that its expression appeared to correlate with disease activity in that weaker staining was observed in the strongly proliferative lining cell layers

(Nikkari *et al.*, 1993). A tenuous link can be made between loss of α6 expression in invasive breast carcinoma and reduced α6 expression in the proliferative rheumatoid synovium. The observation that withdrawal of oestrogen appeared to be the causal factor for downregulation of this subunit in the ZR-75 cell line, and the link between the menopause, and hence loss of sex hormones, and the onset of RA may implicate a role for oestrogen in the maintenance of α6 levels. There is no evidence for such an effect from the data presented in Chapter Five, although the modulation of stimulated α6 levels remains a possibility. This is obviously a very simplistic theory which is likely to incorporate many factors *in vivo* which are absent in the *in vitro* model.

6.4 CONCLUDING REMARKS

RA is a chronic inflammatory disease characterised by a complex scenario of events which culminate in destruction of the joint architecture and a concomitant array of systemic effects. The role for sex hormones in these proceedings is substantiated by the preponderance of females developing the disease, the link between onset and the menopause, and the evidence provided from animal models of arthritis. As the immune system appears to be central to the pathogenesis of RA, and the relationship between the female sex and RA extends to include a range of autoimmune conditions, the obvious target for sex hormones would be that of immune regulation.

There is an ever-increasing body of information regarding the functioning of the immune system, and there are many parameters which could potentially be influenced by sex hormones. Two particular functions which are of direct relevance to the immune system in RA are that of cytokine production and adhesion molecule expression. These processes have the capacity to mediate cellular infiltration and perpetuation in the joint, and the inflammatory reaction and bone and cartilage destruction which ensues. The evidence presented here suggests that the sex hormones, in particular oestrogen, do not directly modulate cytokine or adhesion molecule (integrin) expression by rheumatoid peripheral blood cells or by cells taken from healthy controls.

There are many possible explanations for the failure to demonstrate a sex hormone effect on PBMC in *in vitro* models of immune function. The vast majority of sex hormone effects reported in the literature have resulted from *in vivo* models. PBMC derive from environments with differing hormonal levels which cannot be recreated *in vitro*. In addition, in the *in vivo* situation the coexistence of the different sex hormones, as well as their metabolites and other factors which control sex hormone production and

effects, questions the relevance of *in vitro* models. Other hormones also appear to be important in immune regulation, and it is possible that oestrogen acts via modulating the expression of these proteins. Prolactin is perhaps the most important hormone in terms of immune regulation defined to date (see Introduction, Section 1.8.6). The immuno-potentiating actions of prolactin, and the production of polyamines as a result of prolactin-induced increases in ODC activity, are thought to be involved in the pathogenesis of autoimmune diseases such as SLE and RA. Oestrogen is known to act at the level of the pituitary to stimulate prolactin secretion (Franks, 1983). There is no evidence of an effect of oestrogen on lymphocyte prolactin mRNA expression from experiments reported in this thesis, although a direct effect of oestrogen on immune cells at the translational level cannot be ruled out. Nevertheless, a more likely theory is that oestrogen administered *in vivo* stimulates prolactin release from the pituitary which then modulates immune function directly.

The increasing evidence to suggest that ERs are present on CD8+ T cells, macrophage-like synoviocytes and, as was demonstrated here at the mRNA level, on synovial fibroblasts, indicates that oestrogen has the potential to influence immune function and directly modulate the cells within the joint. The failure to demonstrate ER at the protein level and the detection of only very low levels of ER mRNA, could be due to the small percentage of ER-positive cells present within PBMC. Future investigations using purified cell types are required to provide the information necessary to determine whether this is so, or whether the receptor expression is constitutively low on all cells and hence unlikely to be functional. Distribution of ER on cells within a PBMC population obviously governs their ability to mediate oestrogen response. For example, if monocytes are found to be ER-negative this could explain the lack of effect of oestrogen on PBMC monokine expression, and if CD8+ cells are exclusively ER-positive then assays of immune function, such as cytotoxicity, directed at this cell type would be more applicable. Alternatively, receptors could be present in sufficient amounts to mediate a response, with the failure to elicit a response occurring at a transcriptional level. Thus, an underlying inability to form stable ER/ERE complexes, or a subsequent defect in the transcription of oestrogen-responsive genes, perhaps through the absence of a crucial transcription factor, could explain the hormone-unresponsiveness of immune cells in culture. Differences in EREs could also explain the tissue specificity in oestrogen responsiveness.

The synovial tissue could be considered the target tissue in RA. The gross proliferation of cells, seen within the synovial membrane, and the release of degradative enzymes, for example, are confined to the joint space. Therefore, it is possible that any potential

modulatory effect of the sex hormones in the immune system is localised to the area of inflammatory activity. Indeed, much of the evidence to date demonstrates a direct effect of the sex hormones, especially oestrogen, on bone cells, which is thought to be of importance in the bone disorder, osteoporosis. A closer investigation of ER expression and integrin molecule distribution within the synovial membrane and an examination of the effects of the sex hormones on synovial cells *in vitro*, would provide an interesting successor to the studies carried out as part of this study. In addition, it has been reported that the upregulation of adhesion molecules on the endothelium could facilitate the accumulation of inflammatory cells within the joint. Hence, the endothelial cell provides another possible means of hormonal intervention which would have direct relevance to changes which occur in RA.

There remains a considerable scope for research into the area of immunoendocrinology, and much work is required to unravel the complexities of sex hormone modulation of the immune system. The research carried out as part of this project can only support an indirect role for oestrogen in the periphery, suggesting that this hormone alone is not responsible for the heightened immune response in females. However, immune regulation encompasses a vast spectrum of interactions between cells and mediators, therefore this study is only the tip of the iceberg. A final conclusion that sex hormones act purely indirectly would have important consequences regarding the general perception of sex and immunity. Alternatively, the discovery of a direct role for sex hormones in autoimmune diseases such as RA would clarify many of the complexities in this area and open the door to a host of new therapeutic approaches.

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